A YEAST GENOMIC SCREEN TO IDENTIFY EFFECTORS OF Kir2.1 PLASMA MEMBRANE RESIDENCE

by

Alexander Richard Kolb

B.A. Biology: Health Professions and B.A. Chemistry, Northwestern College, 2006

Submitted to the Graduate Faculty of

The Kenneth P. Dietrich School of

Arts and Sciences in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2013

UNIVERSITY OF PITTSBURGH

DIETRICH SCHOOL OF ARTS AND SCIENCES

This dissertation was presented

by

Alexander Richard Kolb

It was defended on

April 15, 2013

and approved by

Karen M. Arndt, Ph.D., Professor, Biological Sciences

Linton M. Traub, Ph.D., Professor, Cell Biology & Physiology

Ora A. Weisz, Professor, Ph.D., Medicine and Cell Biology & Physiology

Thomas R. Kleyman, Professor M.D., Medicine and Cell Biology & Physiology

Dissertation Advisor: Jeffrey L. Brodsky Ph.D., Professor, Biological Sciences

A YEAST GENOMIC SCREEN TO IDENTIFY EFFECTORS OF Kir2.1 PLASMA MEMBRANE RESIDENCE

Alexander Richard Kolb, PhD

University of Pittsburgh, 2013

Protein quality control is important for the disposal of toxic misfolded proteins. Ion channels represent a class of proteins that are suited for the study of protein quality control within the secretory pathway. The inward rectifying potassium channel, Kir2.1, functions at the plasma membrane in several tissues to maintain membrane potential and osmotic homeostasis, and not surprisingly defects in Kir2.1 lead to a host of diseases. To identify Kir2.1 regulators, a yeast Kir2.1 expression system was established. Although a small population appeared to localize to the plasma membrane, Kir2.1 primarily localized to the ER and a significant fraction was targeted for ER associated-degradation (ERAD). To identify the spectrum of factors that maintain Kir2.1 plasma membrane residence, the expression plasmid was introduced into a potassium transporter deficient strain in which Kir2.1 restores growth on low potassium, and the strain was crossed to the deletion collection to obtain haploid progeny. Among the mutated genes that significantly increased cell growth on low potassium were those encoding members of the Endosomal Sorting Complex Required for Transport (ESCRT). ESCRT also regulated Kir2.1 stability in HeLa cells. These data indicate that Kir2.1 function is controlled by later steps in

secretory pathway quality control, but not ERAD. The screen described represents a model that can be used for other ion channels.

TABLE OF CONTENTS

PREFACE XI

1.0 INTRODUCTION 1

1.1 YEAST AS A MODEL SYSTEM 4

1.1.1 Advantages of the yeast system 4

1.1.2 Yeast to study human disease 5

1.1.3 Limitations of the yeast system 7

1.2 YEAST AS A MODEL SYSTEM TO STUDY RENAL PHYSIOLOGY 8

1.2.1 Ion channels 13

1.2.1.1 Epithelial Sodium Channel (ENaC) 13

1.2.1.2 Inward rectifying potassium channels 15

1.2.1.3 Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) 17

1.2.2 Transporters 21

1.2.3 Water channels 26

1.2.4 Signaling and other molecules 28

1.3 INWARD RECTIFYING POTASSIUM CHANNELS 29

1.3.1 Introduction 29

1.3.2 Kir2.x subfamily 33

1.3.2.1 Kir2.1 33

1.3.2.2 Kir2.1 in yeast 35
1.4 PROTEIN QUALITY CONTROL 36
1.4.1 Endoplasmic Reticulum Associated Degradation (ERAD) 38
1.4.1.1 Recognition by chaperones 38
1.4.1.2 Ubiquitination of ERAD substrates 39
1.4.1.3 Retrotranslocation and degradation 40
1.4.2 Golgi and plasma membrane quality control 41
1.5 ENDOSOMAL SORTING COMPLEX REQUIRED FOR TRANSPORT (ESCRT) 42
1.6 THESIS OUTLINE 46
2.0 A YEAST GENOMIC SCREEN REVEALS ESCRT REGULATES KIR2.1 PLASMA MEMBRANE RESIDENCE 48
2.1 INTRODUCTION 48
2.2 MATERIALS AND METHODS 52
2.2.1 Yeast strains and growth conditions 52
2.2.2 Plasmid construction 59
2.2.3 Genetic screening conditions 60
2.2.4 Indirect immunofluorescence in yeast 61
2.2.5 Biochemical assays 62
2.2.6 Analysis of Kir2.1 in HeLa cells 63
2.2.7 Statistical Analysis 65
2.2.8 Antibodies 65
2.3 RESULTS 66

2.3.1 Kir2.1 is an ERAD substrate 66

2.3.2 A yeast genomic screen identifies regulators of Kir2.1 biogenesis 73

2.3.3 Proteasome and ESCRT-dependent degradation of Kir2.1 in human cells 85

2.4 DISCUSSION 88

3.0 DISCUSSION 93

APPENDIX A 101

APPENDIX B 119

BIBLIOGRAPHY 135

LIST OF TABLES

Table 1: Yeast studies of renal proteins 11

Table 2: Yeast strains 54

Table 3: Oligonucleotide primers 58

Table 4: Genes that when deleted led to increased growth on low potassium in the *trk1Δ trk2Δ* strain 79

Table 5: Expanded list of genes identified in a screen for increased Kir2.1 funciton 101

Table 6: The UPR is induced in yeast strains expressing ENaC. 134

LIST OF FIGURES

Figure 1: The major plasma membrane and intracellular cation transporters in the yeast *S. cerevisiae* 6

Figure 2: A topological and cellular model of the diverse kidney proteins that have been characterized in the yeast system. 10

Figure 3: Kir2.1 topology 32

Figure 4: Protein quality control in the secretory pathway 37

Figure 5: ESCRT functions to produce MVBs 45

Figure 6: Kir2.1 localization in yeast. 68

Figure 7: Kir2.1 degradation is proteasome- and cytosolic chaperone-dependent. 70

Figure 8: Kir2.1 degradation and ubiquitination are E3 ubiquitin ligase dependent. 72

Figure 9: Optimization of Kir2.1 screening conditions 74

Figure 10: *doa10Δ* modestly increases Kir2.1 dependent rescue of *trk1Δ trk2Δ* on low K^+ 76

Figure 11: A genetic screen identifies ESCRT as a major effector of Kir2.1 78

Figure 12: Deletion of *VPS23* increases Kir2.1 dependent rescue of *trk1Δ trk2Δ* on low potassium medium. 81

Figure 13: Affinity tagged Kir2.1 behaves similar to wild type Kir2.1. 82

Figure 14: Kir2.1Δ314-315 is degraded by the 26S proteasome 84

Figure 15: Kir2.1 is degradation is primarily lysosomal dependent and requires ESCRT 86

Figure 16: Ubiquitination of ENaC..... 123

Figure 17: Optimization of the in vitro ubiquitination assay for α-ENaC. 125

Figure 18: Growth assays of *trk1Δ trk2Δ* yeast expressing ENaC mutants..... 130

Figure 19: Sucrose gradient analysis of ENaC 133

PREFACE

It is with great joy that I approach the culmination of my graduate studies and such an accomplishment would not be possible without the direct and indirect contributions of many wonderful people. The person that contributed the most to my studies is my advisor, Dr. Jeffrey Brodsky. Jeff's door has always been open to me to discuss data or career goals, and under his guidance, I learned to think more critically, work more efficiently, speak more clearly, and most importantly develop a greater passion for science. His mentorship led to an American Heart Association pre-doctoral fellowship, peer-reviewed publications, and opportunities to present at national scientific conferences. However, the most significant contribution I received under his mentorship is overcoming stage fright I often have while public speaking. I am indeed thankful to have had an advisor that positioned me for success.

Jeff's entire lab is composed of many quality scientists all of whom I would consider close friends. The lab would not operate as well as it does without the work of Jen Goeckeler-Fried. Not only does she keep the lab stocked and people aware of lab etiquette, she provides a friendly smile and encouragement when needed. The multiple post-doctoral researchers in the lab that I have worked with have been a source of advice from topics concerning experimental design, to career goals. Teresa Buck mentored me during my rotation in the lab, introduced me to the rewards and challenges of working with ENaC, and was always there for advice. Kunio Nakatsukasa taught me to do in vitro ubiquitination assays. Patrick Needham introduced me to

Kir2.1 and he contributed greatly to my research. I received advice and encouragement from Chris Guerriero, Cristy Gelling, Lucia Zacchi, Annette Chiang, Ally O'Donnell and Alex Manos-Turvey multiple times even though their projects didn't always directly relate to mine. I appreciate the fact that they were always so approachable and helpful.

I would like to thank the graduate students who overlapped in their studies with me. As a new graduate student, it was senior members like Shruthi Vembar who encouraged me and challenged me to be a better student. Sandlin Seguin, Sarah Grubb, Joe Tran, and Karen Hecht, who started and finished around the same time, were supportive, helpful, and great friends. I am especially thankful to Karen who was the best baymate one could have. It has been just as exciting forming new friendships with graduate students Mike Preston, Lynley Doonan. I have been greatly impressed with the work of Amy Trotnick who enthusiastically took on the challenges of scientific research. She is a great scientist that has shown she will be successful in her future endeavors.

I have been fortunate to have had a committee composed of Karen Arndt, Linton Traub, Ora Weisz, and Thomas Kleyman, that asks insightful questions and provides helpful suggestions as I completed my studies. I also would like to recognize the PIMB program, a small but impactful group of scientists that exposed me to many different facets of cell biology. The department I was in likewise exposed me to a variety of topics that increased my scientific knowledge. It has been a great place to work full of great people.

Of course there are numerous people not associated with the University whose love, prayers and support encouraged me and gave me a peace and security that allowed me to push on even when things became difficult. My mom, dad, and my siblings, Peter and Laura, have all kept me in touch with my roots. Mitch Hill, and the many people from the YA groups at

Bellefield of which I have been a part. My most meaningful contributor has been my wife, Lydia who was there to encourage and support me as I reach the final goal.

As I look back at the many people that were so involved in my time as a graduate student, I realize how incredibly blessed I am to have been surrounded by the people mentioned who all contributed to my success as a graduate student.

1.0 INTRODUCTION

(Sections of this introduction were previously published in Kolb, A.R., T.M. Buck, and J.L. Brodsky. 2011. Saccharomyces cerivisiae as a model system for kidney disease: what can yeast tell us about renal function? *Am J Physiol Renal Physiol*. 301:F1-11.)

Protein synthesis is a fundamental process required of all living organisms, from bacteria to humans. However, protein synthesis can be inefficient and requires the constant monitoring and turnover of misfolded or over-expressed proteins. A misfolded protein may abnormally present patches that make aberrant contacts with itself or with other proteins. At its worst, misfolded proteins can form aggregates and/or alter protein function or localization, leading to cell toxicity (Dobson, 2003). In order to prevent the toxicity that arises from aggregates and unregulated proteins, the cell possesses multiple forms of protein quality control that act at various points within the cell. Protien quality control prevents misfolded or misregulated proteins from reaching or continuing to function by targeting them for degradation. For example, one way of clearing aggregates is through protein quality control systems like autophagy. During autophagy, portions of the cell are enclosed by a double membrane and targeted to the lysosome/vacuole for degradation (Arias and Cuervo, 2011; Mizushima, 2011). To prevent aggregation, the cell possesses additional protein quality control pathways in the cytosol and secretory pathway (Arvan et al., 2002; Chen et al., 2011; Chhangani et al., 2012). Protein quality

control represents an important step to maintain cell viability as illustrated by the numerous diseases associated with protein quality control disruption or misregulation of proteins (Guerriero and Brodsky, 2012).

In the secretory pathway, protein quality control is essential because approximately one-third of all translated proteins are secretory proteins (Ghaemmaghami et al., 2003) and because many of these proteins are glycosylated, possess disulfide bonds, and/or contain segments that traverse the membrane, which must fold properly so as not to disrupt the membrane (Araki and Nagata, 2011). Failure to properly integrate any of these steps may produce a misfolded protein. Within the ER, aberrant proteins may be targeted to the proteasome for degradation through endoplasmic reticulum associated degradation (ERAD) (Araki and Nagata, 2011; Hebert et al., 2010; Smith et al., 2011; Vembar and Brodsky, 2008). Proteins that pass this initial step of protein quality control are further monitored by protein quality control mechanisms within the Golgi and at the plasma membrane, both of which can target misfolded proteins to the vacuole/lysosome (Apaja et al., 2010; Arvan et al., 2002; Jenness et al., 1997). However, the full complement of protein quality factors that monitor misfolded proteins and how systems at different points within the cell interact with each other is unknown.

A large scale genetic screen to identify quality control factors is lacking. The model system, yeast, are exceptionally suited for large scale genomic studies, and yeast have been used to characterize protein quality control mechanisms within the secretory pathway (Carvalho et al., 2006; Chang and Fink, 1995; McCracken and Brodsky, 1996; Vashist and Ng, 2004; Wang and Ng, 2010). However, using yeast to identify the factors involved in protein quality control pathways has been difficult because disruption of protein quality control pathways does not

necessarily produce easily observable phenotypes. Thus, a protein quality control substrate that is required for cell viability is required.

Ion channels and transporters represent ideal proteins for such a screen. Yeast must maintain proper osmotic and cation homeostasis in a variety of extracellular environments, and they possess multiple ion transporters to perform this function (Cyert and Philpott, 2013). Ion channels are important in human physiology as well, and the loss of ion channel regulation results in many channelopathies due to loss of ionic and osmotic homeostasis (Hubner and Jentsch, 2002; Hubner and Jentsch, 2008; Webster and Berul, 2013). Further, these channels are highly subject to protein quality control since they are transmembrane proteins that often must assemble into multimolecular complexes and they undergo open and closed conformational state changes. Therefore, ion channels and transporters are protein classes that are required for cell viability and subject to protein quality control within the secretory pathway, and an examination of the quality control of two ion channels in yeast is a central focus of this thesis.

In the remainder of the introduction, I focus on the benefits and disadvantages of using yeast as a model system. I then present examples of how yeast has been used to examine renal and cardiac protein function and regulation, with specific emphasis on the Kir2.1 potassium channel. Next, I describe various protein quality control mechanisms that may act on Kir2.1. Finally, I discuss the function of ESCRT, a protein complex utilized by both Golgi and plasma membrane quality control.

1.1 YEAST AS A MODEL SYSTEM

1.1.1 Advantages of the yeast system

Protein quality control in eukaryotes is conserved between yeast and man, and the molecular underpinnings of many protein quality control events were first described in yeast. As a model organism, yeast possess critical advantages. First, the growth of large quantities of cells is rapid and inexpensive, thus aiding the development of biochemical assays. Second, glycosylation and processing, which are important for protein function, occur similarly in yeast and mammalian cells (Lehle et al., 2006). Third, yeast are easy to transform and can be engineered to express heterologous proteins. Fourth, yeast exist in a haploid form or diploid state and recombine genes through homologous recombination. This makes genetic ablation a simple undertaking and allows for genome-wide screens for genetic modifiers of essential processes. Fifth, in comparison to other model organisms, abundant tools are available, either commercially or through the yeast community. These tools include the yeast gene knock-out collection, which includes deletions in every non-essential gene (~85% of all genes) (Winzeler et al., 1999); a hypomorphic allele collection, in which essential yeast genes are placed under the control of a repressible promoter or contain destabilizing sequences in their messages (Breslow et al., 2008); a temperature sensitive mutant collection that includes about half of the ~1000 essential genes (Li et al., 2010); and strains in which each open reading frame is either GFP- or TAP-tagged for localization or purification, respectively (Howson et al., 2005). There is also a wealth of yeast-related resources available online, including the *Saccharomyces* Genome Database, BioGRID (General Repository of Interaction datasets), the Yeast GFP Fusion Localization Database, and

DIP (Database of Interacting Proteins), to name a few. Together, it is relatively simple to design, perform and interpret experiments, and to plan subsequent confirmatory studies.

1.1.2 Yeast to study human disease

Heterologous expression in yeast has been used to study the protein quality control of many human proteins whose function must be tightly regulated. This is particularly true of the many ion channels and transporters which function to maintain proper ion homeostasis within the cell. A reason for this is that yeast, like humans, require ion channels and transporters to maintain osmotic and ion homeostasis required for cell viability (Figure 1). Since yeast are equipped to produce and monitor the biogenesis of protein channels, they also can be used to examine human channels that are heterologously expressed in this organism.

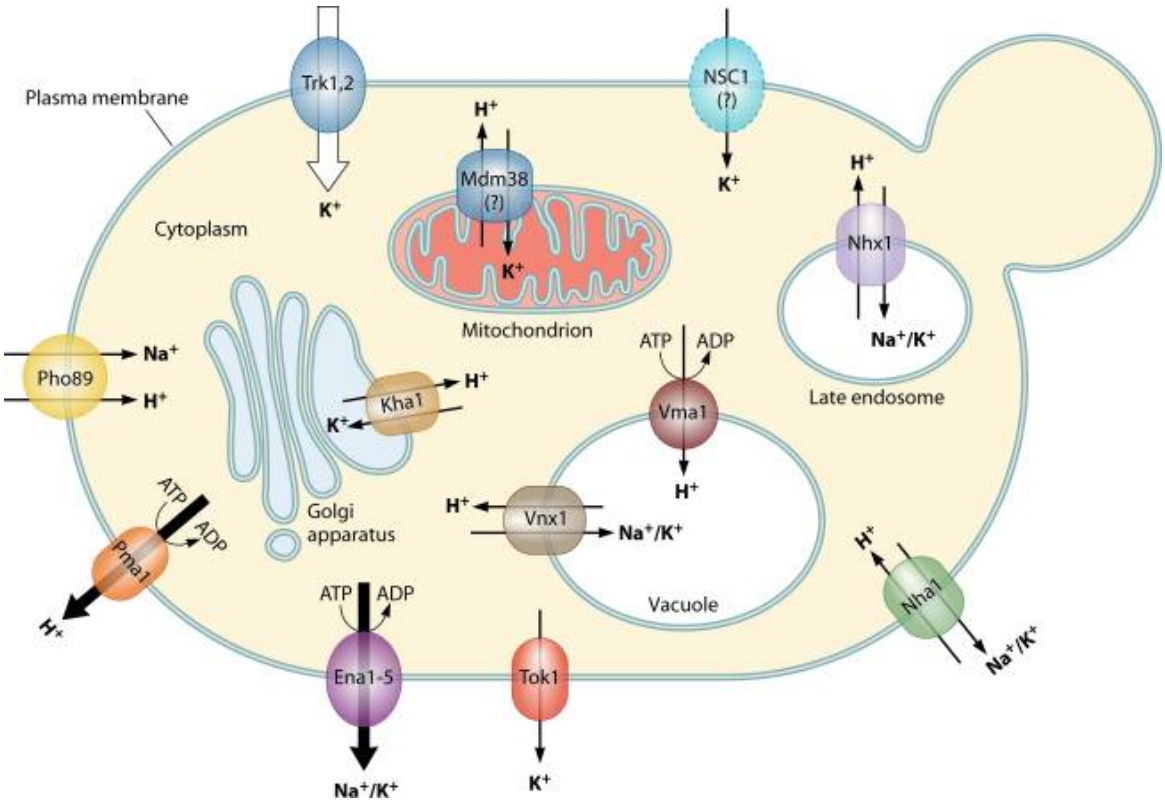


Figure 1: The major plasma membrane and intracellular cation transporters in the yeast *S. cerevisiae*

(Arino et al., 2010)

Misregulation of transporters and channels in humans leads to many channelopathies (Hubner and Jentsch, 2002; Hubner and Jentsch, 2008; Webster and Berul, 2013) and thus represents an important area of study. The kidney is a particularly important site of ion channel function, as it maintains physiological ion and osmotic homeostasis within the blood (Webster and Berul, 2013). In the next section (1.2), I describe how yeast have contributed to a better understanding of channels and transporters in renal physiology. I then shift focus to ion channel function important for heart function. For example, the coordinated action of depolarizing sodium and calcium and repolarizing potassium channels regulate heart contractions (Giudicessi and Ackerman, 2012). One focus of my research is Kir2.1, a potassium channel that participates in the repolarization of the ventricle. Together, regardless of where they function, the mechanisms governing channel biogenesis and regulation are important to understand in order to treat the multiple channelopathies that exist.

1.1.3 Limitations of the yeast system

A discussion of any system is incomplete without mentioning its limitations. Two of the major limitations to studying human physiology in the yeast system are that lab strains of yeast are single cell organisms and that yeast cells are not polarized, except during bud emergence (Johnson et al., 2011). Therefore, yeast cannot be used to model the complicated cell-to-cell interactions that occur within organs. Moreover, studies to examine the residence of proteins to distinct polarized areas in the cell cannot be interpreted in yeast. Nevertheless, this simple system has facilitated studies that have led to the isolation of factors required for the degradation, folding, and transport of critical human proteins. The yeast system has also permitted a robust means to perform structure-function analyses of these proteins, and it has uncovered new

functions and the effects of disease-causing mutants in specific proteins of human physiological relevance. This simplified system can also be utilized to more completely view the essential cellular pathways that impact metabolic and cellular processes.

1.2 YEAST AS A MODEL SYSTEM TO STUDY RENAL PHYSIOLOGY

Approximately 11% of adults age 20 and older (~20 million people in the United States) suffer from some form of chronic kidney-related disease (Levey et al., 2009), and in recent years more than a half-a-million patients in the US were under treatment for end-stage renal disease. Some of these diseases, which specifically affect glomerular and tubular function, are the direct result of defective protein function or the misrouting, misprocessing, and/or degradation of a specific protein. In most cases, however, the molecular basis of disease is unknown or poorly understood.

A number of systems have been employed to study cellular aspects of kidney disease, including mouse models, primary and immortalized tissue culture cell lines, and *Xenopus* oocytes. Mouse or other rodent models for disease are most desirable, but these systems are costly and the generation of mutations is an uncertain and long-term undertaking. Tissue culture systems have been used to successfully model renal epithelia, but genetic manipulation of mammalian cells may not be trivial and there is often debate on which cell type represents the best model. To study ion and water channel physiology and gating, functional assays may be performed in *Xenopus* oocyte expression systems, but biochemical techniques using this system are laborious and limited by the amount of material that can be obtained. While critical discoveries have certainly been made using these systems, there is always a need for faster,

easier, cheaper, and more genetically amenable systems. One such system, and the focus of my thesis, is the baker's yeast, *Saccharomyces cerevisiae*.

In the next section, the techniques, expressions systems, and scientific advances in kidney biology that have been made to date using this system are described as an example that the humble Baker's yeast can indeed tell us a lot about human physiology and disease. I have attempted to highlight the diversity of not only the renal proteins (Fig. 1) that have already been investigated in yeast, but the diversity of techniques this model system offers for studying these proteins (Table I) and the possibility of extending these studies to proteins that reside in other tissues.

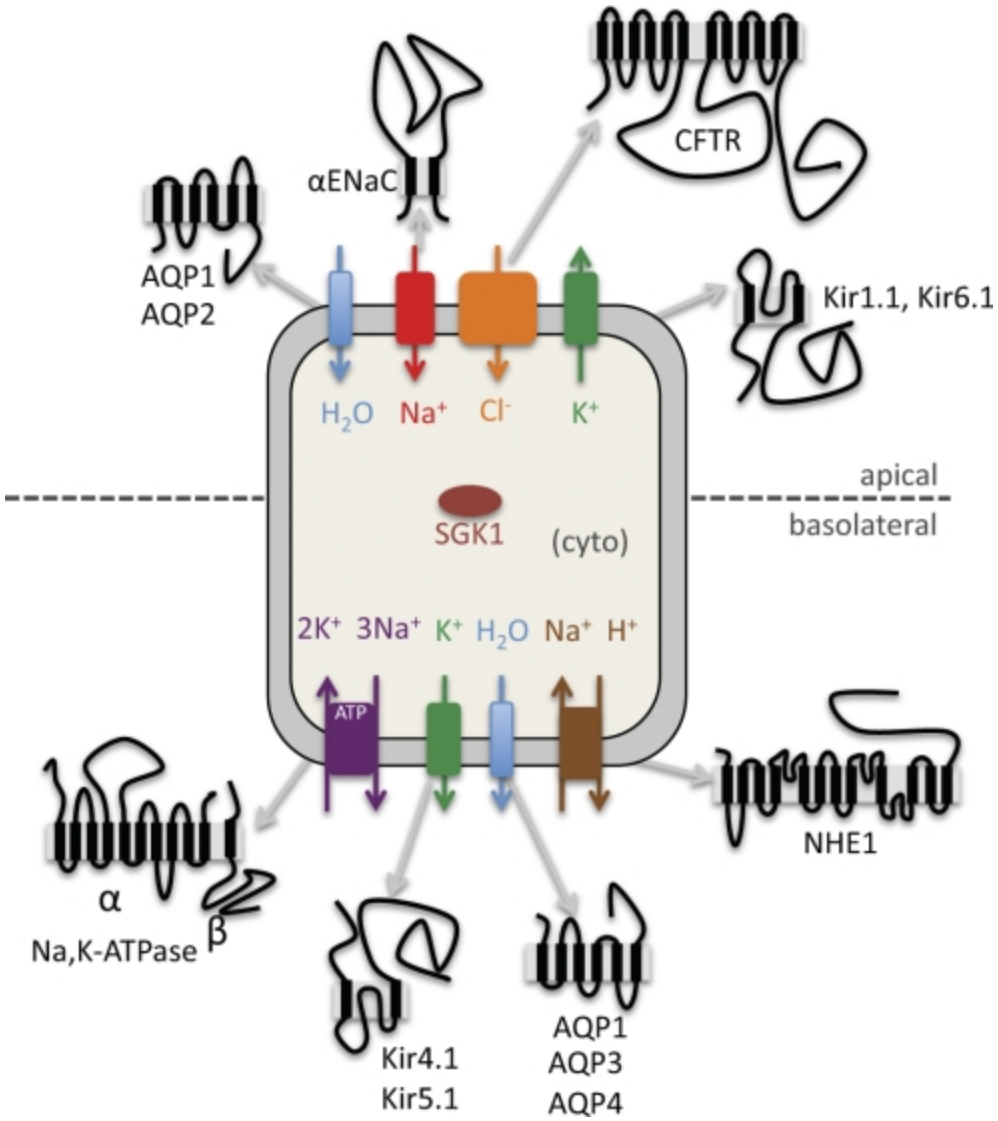


Figure 2: A topological and cellular model of the diverse kidney proteins that have been characterized in the yeast system.

The proteins modeled above are expressed in renal epithelial cells and are localized, as depicted, to the cytosol, apical or basolateral membranes. Please note that these proteins may not be located to the same type of kidney epithelia or region of the kidney nephron.

Table 1: Yeast studies of renal proteins

Protein	Type of Study	Method used	Reference
Ion Channels			
ENaC	Function	Sodium sensitive growth	(Gupta and Canessa, 2000)
	Localization	Membrane preparations	(Gupta and Canessa, 2000)
		Immunofluorescence	(Buck et al., 2010)
	ERAD	Cycloheximide chase	(Buck et al., 2010; Kashlan et al., 2007)
	Characterization	Immunoblotting	(Buck et al., 2010)
		in vitro assay	(Buck et al., 2010)
Kir	Channel Function	Rb^+ flux assay	(D'Avanzo et al., 2010)
		Low potassium rescue	(Haass et al., 2007; Minor et al., 1999; Nakamura and Gaber, 1998; Tang et al., 1995b; Zaks-Makhina et al., 2009)
	Structure	Low potassium rescue	(Minor et al., 1999)
	Small molecule studies	Low potassium rescue	(Zaks-Makhina et al., 2009)
CFTR	Structure/Function	Screen: Rescue of mating phenotype	(DeCarvalho et al., 2002; Teem et al., 1993; Teem et al., 1996)
	Localization	Yeast two-hybrid	(DeCarvalho et al., 2002)
		Immunofluorescence	(Fu and Sztul, 2003; Fu and Sztul, 2009; Kiser et al., 2001; Zhang et al., 2001)
		Membrane preparations	(Fu and Sztul, 2003; Zhang et al., 2001)
		Fractionation	(Kiser et al., 2001; Zhang et al., 2001)
		EM	(Fu and Sztul, 2003; Sullivan et al., 2003)
	ERAD	Cycloheximide chase	(Ahner et al., 2007; Youker et al., 2004; Zhang et al., 2001)
	Characterization	Pulse chase	(Fu and Sztul, 2003; Gnann et al., 2004; Kiser et al., 2001; Lenk et al., 2002)
		Fluorescence microscopy	(Fu and Sztul, 2009)
	Yeast Response	Microarray	(Ahner et al., 2007)
Transporters			
Na,K-ATPase	Function	Rb^+ & Ti^+ occlusion	(Nielsen et al., 1998; Scheiner-Bobis and Farley, 1994)
		Ouabain binding	(Horowitz et al., 1990; Jacobsen et al., 2002; Muller-Ehmsen et al., 2001; Pedersen and Jorgensen, 1992; Pedersen et al., 2000; Pedersen et al., 1998; Pedersen et al., 1996a; Pedersen et al., 1996b; Pedersen et al., 1997a; Pedersen et al., 1997b; Scheiner-Bobis, 2001; Scheiner-Bobis and Farley, 1994; Scheiner-Bobis et al., 1994; Scheiner-Bobis and Schneider, 1997; Scheiner-Bobis and Schreiber, 1999; Xu et al., 2003a)

		ATPase assays	(Horowitz et al., 1990; Jacobsen et al., 2002; Muller-Ehmsen et al., 2001; Pedersen and Jorgensen, 1992; Pedersen et al., 2000; Pedersen et al., 1998; Pedersen et al., 1996a; Pedersen et al., 1996b; Pedersen et al., 1997a; Pedersen et al., 1997b; Scheiner-Bobis, 2001; Scheiner-Bobis and Farley, 1994; Scheiner-Bobis et al., 1994; Scheiner-Bobis and Schneider, 1997; Scheiner-Bobis and Schreiber, 1999; Wang and Farley, 1998; Xu et al., 2003a)
	Structure/Function	β-galactosidase chimera	(Yoon and Lee, 1998)
		yeast two hybrid	(Colonna et al., 1997; Wang and Farley, 1998)
	Yeast Response	Transcription/Translational effects	(Steffensen and Pedersen, 2003; Steffensen and Pedersen, 2006)
	Small Molecule Studies	ATPase assays & ouabain binding	(Hauck et al., 2009; Scheiner-Bobis, 2001; Scheiner-Bobis et al., 1994; Scheiner-Bobis and Schneider, 1997)
Na^+/H^+ antiporter/exchanger	Function	Sodium sensitivity	(Xiang et al., 2007)
		Reconstitution	(Montero-Lomeli and Okorokova Facanha, 1999)
	Localization	Immunofluorescence	(Flegelova et al., 2006; Flegelova and Sychrova, 2005; Xiang et al., 2007)
Na/P_i cotransporter	Function	Low potassium rescue	(Bernhardt et al., 1999)
	Localization	Biotinylation	(Bernhardt et al., 1999)
Aquaporins	Function	Stop flow experiments	(Coury et al., 1998; Kuwahara et al., 1999; Laize et al., 1997; Laize et al., 1995; Mathai and Agre, 1999; Yukutake et al., 2008)
		Protoplast bursting assays	(Pettersson et al., 2006)
		Complementation studies	(Beitz et al., 2006)
	Structure/Folding	Stop flow experiments	(Kuwahara et al., 1999; Mathai and Agre, 1999)
	Disease mutants	Stop flow experiments	(Shinbo et al., 1999)
Signaling and Other Molecules			
SGK1	Function	Complementation studies	(Casamayor et al., 1999; Jacquier and Schneiter, 2010; Sun et al., 2000)
	ERAD	Pulse chase	(Arteaga et al., 2006)
RhGK	Function	Low ammonia rescue	(Marini et al., 2000)
TREH	Function	Complementation studies	(Ouyang et al., 2009)

1.2.1 Ion channels

Ion channel function in the kidney is vital to maintain osmotic and salt homeostasis, and as discussed above, a number of diseases are the direct result of mutations and/or misregulation of kidney-localized ion channels. Ion channels including ENaC (Epithelial Sodium Channel), several members of the Kir (Inward rectifying Potassium Channel) family, and CFTR (the Cystic Fibrosis Transmembrane Conductance Regulator) have been successfully characterized in yeast, an organism that normally lacks these proteins (Figure 2; Table I).

1.2.1.1 Epithelial Sodium Channel (ENaC)

ENaC, a heterotrimeric sodium channel composed of α, β, and γ subunits, is expressed in the kidney, colon, and airway where it functions to maintain osmotic homeostasis (Snyder, 2002; Soundararajan et al., 2010). ENaC is responsible for final sodium absorption in the distal nephron. Thus, ENaC function and levels are tightly regulated, and either gain- or loss-of-function mutations in ENaC alter sodium homeostasis in the kidney, resulting in hypertension (Liddle's Syndrome), or hypotension (Pseudohypoaldosteronism Type I), respectively. In addition, common polymorphisms in the genes encoding ENaC subunits may affect blood pressure variation in the population as a whole (Jin et al., 2010).

To examine how ENaC is regulated during biosynthesis, the three ENaC subunits were expressed individually in yeast. Immunofluorescence microscopy was used to determine that ENaC localized primarily to the ER (endoplasmic reticulum), although other immunostaining regions were observed that may represent secretory vesicles and/or the plasma membrane (Buck et al., 2010; Gupta and Canessa, 2000). The primary ER residence of ENaC is consistent with

the fact that <20% of the channel resides at the plasma membrane in epithelial cells (Hanwell et al., 2002; Prince and Welsh, 1998; Staub et al., 1997; Valentijn et al., 1998; Weisz and Johnson, 2003). This makes yeast an ideal system to monitor early events during ENaC biogenesis, such as ER-associated degradation (ERAD) (see section 1.4.1) (Weisz and Johnson, 2003).

ERAD is a quality control pathway exemplified by the chaperone-mediated recognition of misfolded proteins within the ER and the subsequent targeting of these aberrant proteins for ubiquitination and degradation by the cytosolic 26S proteasome; notably, ERAD was first discovered in yeast and the components and basic elements of this pathway are completely conserved (Goeckeler and Brodsky, 2010; Vembar and Brodsky, 2008). Consistent with studies in vertebrate systems (Malik et al., 2006; Malik et al., 2001; Staub et al., 1997; Valentijn et al., 1998), ENaC degradation was slowed in yeast strains with defects in the proteasome pathway when analyzed by a cycloheximide chase assay (Buck et al., 2010; Kashlan et al., 2007). Using recently developed biochemical techniques, it was then determined that the ER resident E3 ubiquitin ligases, Hrd1 and Doa10, append ubiquitin onto the ENaC subunits and facilitate their degradation (Buck et al., 2010). Interestingly, the extent of ubiquitination and stabilization in the E3 mutant strains varied amongst the subunits. This result supports data from other systems suggesting that the three subunits are differentially regulated (Canessa et al., 1994a; Hughey et al., 2003; Mueller et al., 2010; Staub et al., 1997).

Because molecular chaperones aid in secretory protein folding and select misfolded proteins for ERAD, the chaperone requirements for ENaC degradation were also assessed in yeast. Cycloheximide chase assays revealed that the small heat shock proteins, Hsp26 and Hsp42, facilitate the degradation of α-ENaC (Kashlan et al., 2007), and the ER lumenal Hsp40 chaperones, Scj1 and Jem1, promote the degradation of each of the three ENaC subunits. By

employing reagents obtained from wild type and mutant strains in defined cellular and *in vitro* assays, the Hsp40s were shown to function prior to substrate ubiquitination (Buck et al., 2010). The results obtained from the yeast studies were then validated in vertebrate cells. Specifically, overexpression of the mammalian homologs of the small heat shock proteins or the Hsp40s in *Xenopus* oocytes accelerated ENaC degradation, and their expression decreased the amiloride-sensitive ENaC current and ENaC residence at the plasma membrane (Buck et al., 2010; Kashlan et al., 2007). These studies indicate that yeast can provide a means to identify factors involved in ion channel biogenesis, and that results in this system can be translated into higher cell types.

Earlier, another yeast expression system for ENaC, consisting of an inducible αβENaC concatamer, was established (Gupta and Canessa, 2000). Immunoblotting of secretory vesicles and plasma membrane preparations confirmed that ENaC traffics to the plasma membrane, as it does in epithelial cells (see above). Interestingly, ENaC expression led to defective cell growth when yeast were incubated on high (1M) sodium-containing media (Gupta and Canessa, 2000). These data suggested that ENaC is active in yeast, which sets-the-stage for genome-wide studies to identify and characterize additional regulators of ENaC function.

1.2.1.2 Inward rectifying potassium channels

A group of ion channels unrelated to those discussed have also been expressed in yeast. The Kir family is composed of seven subfamilies (Kir1-7) that share ~60% sequence homology and ~40% sequence identity within subfamilies (de Boer et al., 2010). Several members of the Kir potassium channel family are expressed in the kidney, including Kir1.1, Kir4.1, Kir5.1 and Kir6.1 (de Boer et al., 2010; Hibino et al., 2010). Kir1.1 (also known as ROMK) functions at the apical membrane and Kir4.1 and 5.1 function at the basolateral membrane of polarized epithelial cells. These channels maintain potassium homeostasis and provide potassium to the

Na^+-K^+-$2Cl^-$ co-transporter and to the Na^+-K^+-ATPase, thus contributing to the cellular flux of sodium and chloride. Mutations in Kir1.1 result in Type 2 Bartter syndrome, which is characterized by potassium and sodium wasting (Hibino et al., 2010), and mutations of Kir4.1 were recently shown to cause EAST/SeSAME syndrome, which also gives rise to a Bartter-like phenotype (Bockenhauer et al., 2009; Scholl et al., 2009). Although Kir6.1's role in the pancreas is better characterized, Kir6.1 is an ATP-sensitive potassium channel, and resides with Kir1.1 and CFTR on the apical membrane of renal epithelial cells (Hibino et al., 2010; Ruknudin et al., 1998). Kir6.1 is also localized to the mitochondria in the kidney, where it may act as a mitochondrial potassium channel (Ng et al., 2010). While there are insignificant levels of other Kir family members in the kidney, all family members are closely related. Therefore, studies that involve Kir homologs that reside in other tissues may provide insights into the function of renal Kir family members.

Multiple Kir proteins have been expressed in yeast. In fact, ten of eleven Kir proteins, representing every Kir subfamily, have been expressed in this organism and traffic to the plasma membrane, as they do in mammalian cells. Some of these channels were purified from yeast, reconstituted into proteoliposomes, and shown to have channel activity using a Rb^+ flux assay (D'Avanzo et al., 2010).

Another strategy to study channel function in yeast is to express Kir channels in a strain deleted for the genes encoding two endogenous plasma membrane-resident potassium transporters, Trk1 and Trk2. This strain is inviable when grown on low potassium media, but cell growth is rescued upon the expression of a foreign, functional potassium channel that resides at the plasma membrane. Kir2.1 was the first Kir channel expressed in this strain background

and restored cell growth on low potassium-containing media (Tang et al., 1995b). This system was then optimized for genetic screens (Nakamura and Gaber, 1998).

For example, a genome-wide SGA (synthetic gene array) screen was used to isolate factors that impact the trafficking of Kir3.2. Seven genes that affected the protein's residence at the plasma membrane were identified, including: COPII cargo receptors, which mediate the transport of specific proteins from the ER to the Golgi (*ERV25*, *EMP24*, and *TED1)*, a COPII vesicle packaging chaperone (*ERV14*), a fatty acid elongase (*SUR4*), a GPI inositol deacylase (*BST1*) and a regulatory subunit of mannosyl-transferases (*CSG2*) (Haass et al., 2007). Each of these factors plays a designated role in the secretory pathway. Screens in Kir2.1-expressing yeast were also employed to uncover small molecule inhibitors of Kir and to define how the protein's transmembrane domains are organized and contribute to channel function (Minor et al., 1999; Zaks-Makhina et al., 2004; Zaks-Makhina et al., 2009). As described below, I performed a screen for Kir2.1 plasma membrane regulators (see Chapter 2).

In another example, an expression system for Kir6.1 was established, and like other Kir family members the channel rescued the growth of *trk1Δtrk2Δ* mutant yeast on low potassium-containing media. Mutated residues in Kir6.1 that ablated ATP sensitivity and trafficking in *Xenopus* oocytes were then scored as gain or loss-of-function mutations in this system (Graves and Tinker, 2000). In principle, monitoring the growth of *trk1Δ trk2Δ* cells on low potassium-containing media should facilitate continued studies on the function, trafficking, and regulation of any potassium channel that can be expressed in yeast.

1.2.1.3 Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)

The structure, function, biogenesis, and regulation of a third ion channel, CFTR, have also been extensively examined in the yeast system (Table 1). Although its specific activity in

the kidney has not been well characterized, CFTR function in airway cells and in tissue culture has been extensively studied because mutations in CFTR destabilize the protein and result in cystic fibrosis. The most common disease-causing mutation is a deletion of phenylalanine at position 508 (Belcher and Vij, 2010; Cheng et al., 1990; Pilewski and Frizzell, 1999). Although kidney pathology is not associated with cystic fibrosis, it has been proposed that as patient survival increases due to improved treatment of the airway pathology, a renal pathology may become apparent (Soulsby et al., 2009).

CFTR is a chloride channel of the ATP-binding cassette (ABC) transporter family, consisting of 12 transmembrane domains and containing two prominent cytoplasmic loops. The first loop harbors a nucleotide binding domain (NBD1) and a regulatory domain, and the second loop contains a second NBD (NBD2). In the active form CFTR's regulatory domain is phosphorylated and the NBDs are in the ATP bound state (Csanady et al., 2010; Gadsby, 2009). The most common cystic fibrosis-causing mutation, ΔF508, is located near the end of NBD1, and is thought to affect the docking of this domain onto the transmembrane domains (Serohijos et al., 2008). Consequently, the mutation severely affects CFTR folding in the ER, such that the protein is targeted for ERAD (Jensen et al., 1995; Ward et al., 1995). In some cell types, a significant fraction of the wild type protein is trapped in the ER and degraded by the ERAD pathway. However, improvements in the folding environment—by altering chaperone levels or by reduced temperature (Denning et al., 1992)—rescue the folding defect such that the protein can escape ERAD and function at the plasma membrane. Thus, there is a profound need to better define the factors that play a role in the folding and degradation of CFTR, which might then become therapeutic targets.

One approach that was successfully used to explore the protein-folding pathway of CFTR was to use a chimera in which the NBD1 of the yeast ABC transporter, Ste6, was replaced with the NBD1 from CFTR. Ste6 is required for the successful mating between haploid strains in yeast, and the expression of the Ste6-CFTR-NBD1 chimera supported this event. However, mating efficiency was reduced when the cystic fibrosis causing mutation, ΔF508, was introduced into NBD1. The system was then used to identify suppressor mutations that restored function. Strikingly, the mutations also corrected the folding and trafficking defect of full-length ΔF508 CFTR when expressed in mammalian cells (DeCarvalho et al., 2002; Teem et al., 1993; Teem et al., 1996). Even though the nature of the defect in the Ste6-CFTR-NBD1(ΔF508) chimera was subsequently reinterpreted (Paddon et al., 1996), these studies exemplified how yeast could be used to uncover determinants within a domain that permit the proper folding and trafficking of a disease-causing mutant protein.

A yeast expression system for full length CFTR has also been used to define how the protein is targeted for ERAD. A variety of biochemical and cellular assays showed that CFTR resides primarily in the ER (Kiser et al., 2001; Sullivan et al., 2003; Zhang et al., 2001). Cycloheximide and pulse-chase experiments in yeast strains with mutations or deletions in ER-associated factors demonstrated that CFTR degradation is dependent on the proteasome, the ER localized E2 ubiquitin-conjugating enzymes, Ubc6 and Ubc7, and E3 ubiquitin ligases, Hrd1 and Doa10 (see above), and the Cdc48 complex, which is required to extract ubiquitinated proteins from the ER and present them to the proteasome (Gnann et al., 2004; Kiser et al., 2001; Lenk et al., 2002; Nakatsukasa et al., 2008; Zhang et al., 2001). The mammalian homolog of Cdc48, p97 or VCP, is also important for degrading CFTR in higher cells (Vij et al., 2006). Yeast chaperones that contribute to the degradation of immature forms of CFTR include the cytosolic

Hsp40s (Ydj1 and Hlj1) and an associated Hsp70 (Ssa1), which together help present misfolded ER membrane proteins to the ubiquitination machinery. Interestingly, yeast Hsp90 prevented the aggregation of purified NBD1 and aided the folding of CFTR (Youker et al., 2004). The ability of Hsp90 to fold CFTR has been confirmed in higher cell types (Loo et al., 1998; Wang et al., 2006).

To more globally identify factors involved in CFTR biogenesis, a microarray screen analyzed the yeast transcriptome in CFTR expressing cells, with the underlying assumption that factors required for CFTR degradation would be upregulated. From this effort, the small heat shock proteins, Hsp26 and Hsp42, were found to be upregulated and their deletion had a profound effect on CFTR degradation in yeast. In HEK293 cells, a mammalian Hsp26/Hsp42 homolog preferentially selected ΔF508 over wild-type CFTR for degradation (Ahner et al., 2007). This result indicated that the yeast system could be used to uncover a component that can be specifically modulated to rescue the biogenesis of the disease-causing protein in higher cells.

Of note, CFTR degradation in yeast may occur in discrete ER microdomains, termed ER-associated complexes (ERACs) (Huyer et al., 2004a), which are maintained by the COPII/SAR1 vesicle transport machinery (Fu and Sztul, 2003). Real-time fluorescence microscopy of an inducible EGFP-CFTR construct indicated a diffuse ER localization for the protein that was either degraded immediately or accumulated in distinct foci. Using fluorescence recovery after photobleaching technology, the authors determined that the CFTR population in the foci was not as mobile as the CFTR in the diffuse ER pool, and the former pool appeared to be degraded via autophagy (Fu and Sztul, 2009). Links between autophagy and defects in CFTR function, at least under some conditions, were recently reported (Luciani et al., 2010).

1.2.2 Transporters

Osmotic homeostasis in kidney epithelia depends upon the coordinated efforts of ion channels and a diverse group of transporters. For example, the ubiquitously expressed Na^+-K^+-ATPase imports two K^+ ions for every three Na^+ ions exported, and is composed of an α, β, and γ subunit (Kaplan, 2002). The α and β subunits are sufficient to form an active channel while the γ subunit regulates activity. There are three α subunit isoforms and four β-subunit isoforms, but the α_1 and β_1 subunit isoforms are the major isoforms expressed in the kidney. In kidney epithelia, the Na^+-K^+-ATPase functions at the basolateral membrane and provides the electrochemical gradient required for Na^+ transport at the apical membrane, which is mediated by other ion channels, such as ENaC. Na^+-K^+-ATPase function in the kidney has been linked to some forms of hypertension and has thus been targeted for therapeutics (Ferrari et al., 2003; Lingrel et al., 1994). The Na^+-K^+-ATPase also functions in the heart and has been targeted for the treatment of congestive heart failure through the use of cardiac glycosides, such as digoxin, which inhibit the transporter (Francis, 2008; May and Diaz, 2008). Although not used clinically, another cardiac glycoside, ouabain, has been utilized extensively to characterize the function of the Na^+-K^+-ATPase because each ouabain molecule binds one holoenzyme and inhibits pump activity (Lingrel, 2010).

Yeast, unlike animal cells, lack the Na^+-K^+-ATPase, and can therefore be used to characterize its activity. Yeast compensate for the expression of an exogenous transporter by derepressing Gcn4, a transcription factor that mediates a response to nutrient starvation (Steffensen and Pedersen, 2003; Steffensen and Pedersen, 2006). To establish a yeast expression system for the Na^+-K^+-ATPase, a sheep α subunit and a canine β subunit were individually expressed (Horowitz and Farley, 1988). The resulting transporter exhibited potassium-sensitive

ouabain binding and ouabain-sensitive ATPase and *p*-nitrophenylphosphatase activities, as in mammalian cells (Horowitz et al., 1990). This system was then adapted to study the activities of the many possible α - β isoform combinations. This was accomplished by placing each subunit isoform on plasmids with different selectable markers (Pedersen and Jorgensen, 1992). For example, each of the human α isoforms with the β_1 isoform were expressed in yeast and each assembled enzyme exhibited similar potassium-sensitive ouabain binding affinities and activities, as observed in mammalian cells (Muller-Ehmsen et al., 2001). Since yeast lack endogenous Na^+-K^+-ATPase activity, the data are more easily interpreted.

The yeast system has also contributed to structure-function analyses of the Na^+-K^+-ATPase. In one study, truncated ATPase subunits were fused to a β-galactasidase reporter, which is only functional in the cytoplasm, and therefore allows one to determine transmembrane segment orientation. It was found that the α subunit contains ten transmembrane segments while the β subunit contains one transmembrane segment, consistent with results from other studies (Fiedler and Scheiner-Bobis, 1996; Kaplan, 2002). The yeast system also helped to establish that the α and β subunits constitute minimal transporter activity (Scheiner-Bobis and Farley, 1994). Further, the yeast system and yeast two-hybrid assays were used to identify regions within the α and β subunit that contribute to subunit-subunit association: A 63 amino acid tract (E63-D125) in the extracellular loop of the β subunit and the amino acid tract SYGQ along with V904, T890, and C908 in the extracellular loop between the 7^{th} and 8^{th} transmembrane segment of the α subunit were shown to be required to mediate intermolecular associations (Colonna et al., 1997; Wang and Farley, 1998). Yeast two-hybrid assays were also employed to provide evidence that the α subunits may self-associate via the first cytoplasmic loop (Colonna et al., 1997; Yoon and Lee, 1998).

The Na^+-K^+-ATPase cycles through ATP-bound, ADP-bound, and nucleotide-free conformations, but the residues that mediate this cycle and the contributions of bound ions during the cycle were at first poorly understood. To this end, mutated forms of the enzyme, such as one containing the phosphorylation site mutant, D369N, and cation binding site mutants in the α subunit were expressed, assembled, and assayed using ouabain binding and ATPase activity assays in yeast (Pedersen et al., 1996a). As a result of these efforts, phosphorylation at D369 was shown to be critical for a major conformational change of the K^+-bound enzyme (Pedersen et al., 1996b; Pedersen et al., 1997a), and the D804, D808, E327, and E779 residues were found to coordinate sodium and potassium ions as the transporter changes conformation (Jorgensen et al., 1997; Nielsen et al., 1998; Pedersen et al., 1997b). Multiple, other residues were isolated that affect the transporter's ATPase activity (Jacobsen et al., 2002; Scheiner-Bobis and Schreiber, 1999; Xu et al., 2003a). For example, mutations in residues 691 and 708-714 are important for Mg^{2+} binding and D369 phosphorylation (Jorgensen et al., 2001; Su and Scheiner-Bobis, 2004). In addition, mutations in this region (amino acids 708-720) induce the unfolded protein response (UPR) when expressed in yeast at higher temperatures, which indicates that the mutations likely affect protein stability (Jorgensen and Pedersen, 2001). In fact, mis-assembly of the Na^+-K^+-ATPase is known to trigger its degradation through the ERAD pathway in mammalian cells (Beguin et al., 2000), which is consistent with the observed UPR induction upon the expression of the mis-assembled protein in yeast.

As described above for the Kir proteins, drugs that target the Na^+-K^+-ATPase can be identified and characterized in yeast. The yeast expression system was employed to show that the Na^+-K^+-ATPase is the target of palytoxin and sanguarine (Scheiner-Bobis, 2001; Scheiner-Bobis et al., 1994), and that the activity of palytoxin does not depend on a catalytically active

enzyme (Scheiner-Bobis and Schneider, 1997). In addition, cardiac glycosides, such as ouabain, were found to have different isoform specificities (Hauck et al., 2009). Finally, the purified, detergent-solubilized enzyme from yeast membranes was used to validate the use of electrochemical dyes that can report on Na^+-K^+-ATPase activity *in vitro* (Habeck et al., 2009).

A yeast expression system for another transporter, the Na^+-H^+ antiporter, has also been developed. Na^+-H^+ antiporters are widely expressed and help maintain the intracellular pH, which is important for a variety of cellular functions, including cell division (Malo and Fliegel, 2006). There are two Na^+-H^+ antiporter families and alterations in the activities of these enzymes may also affect blood pressure (Malo and Fliegel, 2006; Xiang et al., 2007). The Na^+/H+ exchanger (NHE) is the first family and there are nine paralogs in humans (Malo and Fliegel, 2006). The second group is the Na^+/H^+ antiporter NHA family, which is characterized by the presence of a shorter C-terminal tail and is encoded by two genes in mammals, NHA1 and NHA2, and one homolog in yeast (Xiang et al., 2007). In the proximal tubule of the kidney, Na^+-H^+ antiporters contribute to sodium reabsorption (Bobulescu and Moe, 2009).

When NHE1 was expressed in yeast, it localized primarily to the ER as opposed to the plasma membrane; however, the protein was active after its reconstitution into proteoliposomes (Montero-Lomeli and Okorokova Facanha, 1999). When NHE2 and NHE3 were expressed from a strong promoter, the salt tolerance of yeast lacking endogenous sodium transporters increased slightly in spite of the fact that most of the transporters remained in the ER (Flegelova et al., 2006; Flegelova and Sychrova, 2005). However, a decrease in the amount of the E3 ubiquitin ligase, Rsp5, increased the number of channels at the plasma membrane (Flegelova et al., 2006). Rsp5 is the yeast homolog of the mammalian ubiquitin ligase, Nedd4-2, which is required for the endocytosis and lysosomal degradation of ENaC (Kabra et al., 2008; Wiemuth et al., 2007; Zhou

et al., 2007) and possibly CFTR (Caohuy et al., 2009) after retrieval from the plasma membrane; therefore, these data suggest that later steps during the secretion, recycling, and quality control of renal proteins can be characterized in yeast. Moreover, and consistent with these data, a related E3, Nedd4-1, was recently shown to play a role in mediating NHE1 endocytosis in mammalian cells (Simonin and Fuster, 2010).

Two human NHA genes were also characterized in yeast. NHA1 and 2 rescued growth on high sodium media when expressed in a strain deleted for endogenous sodium transporters, including yeast NHA1 (Xiang et al., 2007). Rescue required plasma membrane residence. These data further emphasize the possibility that yeast may prove to be a new model to identify factors that impact Na^+-H^+ antiporter stability and function.

Another manner in which yeast can be leveraged to explore renal protein biogenesis and structure-function relationships is to express the protein in a yeast strain in which the homolog has been deleted, leaving only the mammalian protein to function in its place. This is analogous to the ability of the human Kir proteins to support the growth of *trk1Δtrk2Δ* yeast on low potassium-containing media (see above). The Na^+-phosphate cotransporter is expressed in many epithelial cells where it functions to maintain phosphate homeostasis, and defects in transporter function lead to diseases such as X-linked hypophosphatemia and autosomal-dominant hypophosphatemic rickets (Biber et al., 2009; Virkki et al., 2007). As anticipated, the wild-type cotransporter, but not an inactive mutant form of the protein trafficked to the plasma membrane and rescued the growth of yeast lacking the high affinity phosphate transport system (Bernhardt et al., 1999). These data further support the ability of yeast to properly fold and secrete renal proteins.

1.2.3 Water channels

The kidney plays a critical role in maintaining water homeostasis, and this role depends on the function of aquaporin (AQP) water channels. AQPs are six transmembrane, ~30kDa proteins that tetramerize (Noda et al., 2010). Humans express 13 AQPs that are divided into three families based on their channel selectivity for water and/or other solutes. Seven of the AQPs are expressed in the kidney, AQP1, AQP2, AQP3, AQP4, AQP6, AQP7, and AQP11. Mutations in two of these channels have been identified that lead to urine concentration defects (AQP1) and nephrogenic diabetes insipidus (AQP2). Four of the renal aquaporins (AQP1-4) have been successfully expressed in yeast.

AQP1 was the first human aquaporin to be expressed in yeast (Laize et al., 1995). The AQP1 expression system was used to assess channel function by isolating AQP1-containing vesicles and subjecting them to stop-flow experiments, which spectrophotometrically measure changes in vesicle size in response to hyperosmotic conditions. In order to increase the signal-to-noise, these experiments were carried out in a mutant yeast strain, *sec6-4,* which has an exocytosis defect that results in the accumulation of intracellular vesicles. To further improve the sensitivity of this assay, some experiments were performed by first detergent solubilizing and purifying the AQPs from yeast, and then reconstituting the channels into synthetic proteoliposomes, in which the stop-flow experiments could be performed. This approach permitted tighter control of the AQP:lipid ratio (Coury et al., 1998; Laize et al., 1997). The effects of known channel inhibitors, such as $CuSO_4$, $AgNO_3$, TEA, and pCMBS, could then be assayed (Laize et al., 1995; Pettersson et al., 2006). In fact, it was found that $HgCl_2$ inhibits AQP4, contrary to previous studies in oocytes (Yukutake et al., 2008).

Yeast expression systems for AQPs have also been used to study channel selectivity as well as the structure-function relationship of channels that were subjected to site-directed mutagenesis. To compare the properties between AQP family members, a protoplast-bursting assay was carried out. This study determined that AQP1 has a larger water conductance than AQP3, 5 or 9 (Pettersson et al., 2006). Similar to what was shown for AQP1 isolated from red blood cells, AQP2 appears to be water-selective as urea, glycerol, or formamide were not transported by the channel (Coury et al., 1998; Zeidel et al., 1995). Additional studies of AQP pore selectivity focused on different AQP classes, which are defined by their selectivity for solutes and the NPA amino acid motif. Class I aquaporins are primarily water selective, class II aquaporins are both water-permeable and are permeable to small neutral solutes, such as glycerol and urea, and class III aquaporins are the least selective. It was found that class I aquaporins were impermeable to H_2O_2 whereas the class II aquaporin, AQP3, was H_2O_2 permeable (Bienert et al., 2007). Studies investigating the properties of class III aquaporins found that AQP7 and AQP9 transport arsenite, providing a hint as to the route through which arsenic may enter cells (Liu et al., 2002). The residues that mediated selectivity within the pore region, H180 and F56A, were defined for AQP1 (Beitz et al., 2006). In another investigation, cysteine mutations within the pore-forming domains of AQP1 were examined. These mutations abolished water channel function, and sucrose gradient analyses established that the absence of channel function was due to an inability of the mutant channel to tetramerize. Thus, the residues that are critical for AQP assembly and selectivity could be defined (Mathai and Agre, 1999).

Another important application of the yeast system was to characterize ten AQP2 mutations that are known to cause nephrogenic diabetes insipidus. These mutants fell into three categories: those that are functional, those that are partially functional, and those that are

completely inactive (Shinbo et al., 1999). Together, these studies have contributed significantly to our overall understanding of several aspects of AQP biology, including differences between AQP family members, the structure-function relationships regarding the selectivity filter, the importance of intermolecular contacts required for tetramer formation, and a molecular characterization of disease-causing mutants.

1.2.4 Signaling and other molecules

The yeast expression system has also been used to characterize kidney-resident proteins that are involved in signaling and other activities (Table 1). One of these proteins is SGK1, a cytosolic kinase of the AGC family (Loffing et al., 2006). SGK1 is activated in response to a variety of stimuli, including aldosterone. Aldosterone, among other stimuli, transcriptionally regulates SGK1, which then either directly or indirectly regulates various channels and transporters, including ENaC, the Na^+,K^+-ATPase, Kir1.1, and the Na^+-K^+-$2Cl^-$ co-transporter (Lang et al., 2009; Loffing et al., 2006), each of which was discussed above.

Yeast encode two kinases, Ypk1 and Ypk2, whose catalytic domains share 55% sequence identity with the catalytic domain of SGK1. When expressed in yeast, SGK1 was able to restore the viability of yeast lacking the genes encoding both Ypk1 and Ypk2, making SGK-dependent functional assays possible in this mutant background. Like the ion channels discussed in the preceding section (i.e., ENaC and CFTR), mutant yeast strains were then used to identify factors that modulated SGK1 levels. It was discovered that SGK1 turn-over required the E2 ubiquitin-conjugating enzymes, Ubc6 and Ubc7, the E3s Hrd1 and Doa10, and the proteasome (Arteaga et al., 2006). Therefore, even though SGK1 is a cytosolic protein, it can interact with the cytoplasmic face of the ER and is degraded by the ERAD pathway. By analogy, PDK1 is a

constitutively active kinase which regulates AGC kinases, including SGK1 (Bayascas, 2008). Yeast encode two PDK1 homologs, Pkh1 and Pkh2, and the expression of human PDK1 can rescue the growth of yeast strains in which the genes encoding Pkh1 and Pkh2 are deleted (Casamayor et al., 1999; Loffing et al., 2006). This result indicates that the requirements for the functional expression of PDK1 can ultimately be defined in the yeast system.

The characterization of two other renal proteins, RhGK and TREH, has benefited from the development of yeast expression systems. RhGK (Rh glycoprotein kidney) is a poorly characterized protein, but by expressing RhGK in yeast it was demonstrated that it functions as an ammonium transporter (Marini et al., 2000). TREH, a glycoprotein expressed in small intestines and renal brush borders, is responsible for metabolizing trehalose to glucose. The presence of TREH in the urine is a marker of kidney damage. Yeast have three trehalases, ATH1, NTH1 and NTH2, each possessing a unique function *in vivo*. Gene complementation studies in yeast deleted for the endogenous trehalases demonstrated that the human trehalase is not required for general metabolism (i.e., metabolizing trehalose to glucose), but may instead be a stress response protein that might be important for renal function (Ouyang et al., 2009).

1.3 INWARD RECTIFYING POTASSIUM CHANNELS

1.3.1 Introduction

There are seven Kir channel subfamilies (Kir1-7) consisting of 15 proteins and these play critical roles in the function of several tissues including the kidney and heart (Hibino et al., 2010). These are named for their ability to conduct more potassium ions into a cell at potentials negative

to the equilibrium potential, but permit less flow at potentials positive to the equilibrium potential, which is contrary to that predicted by the Nernst equation (Hagiwara and Takahashi, 1974; Noble, 1965). This is accomplished by magnesium and polyamines blocking the intracellular side of the pore under potentials positive to the equilibrium potential and the release of the molecules under potentials negative to the membrane potential (Lopatin et al., 1994; Matsuda et al., 1987). Kir channels are also activated by phosphatidylinositol 4,5-bisphosphate, which limits channel activity to the plasma membrane where phosphatidylinositol 4,5-bisphosphate is most abundant (Huang et al., 1998; Lopes et al., 2002). These combined characteristics allow Kir channels to serve a variety of functions including restoring resting membrane potentials in cardiac, neuronal or muscle cells and reabsorbing potassium in the kidney (see section 1.2.1.2).

In order for the channels to perform their critical functions, they must assemble into tetramers. Tetramerization can be either homomeric or heteromeric. While heteromeric tetramerization is usually associated within members of the same subfamily, there are cases of heteromeric tetramers between families such as is seen between Kir4.1 and Kir5.1 (Konstas et al., 2003; Lourdel et al., 2002; Preisig-Muller et al., 2002). As monomers, Kir channels are likely unstable and highly subject to protein quality control. The transmembrane segments and termini in the monomers have been implicated in the formation of tetramers (Fink et al., 1996; Tinker et al., 1996), and as monomers these contact sites may be recognized as being misfolded by the protein quality control machinery. In addition, the hydrophilic face of the pore (Minor et al., 1999) would be exposed to the membrane. Thus, Kir channel assembly represents an additional challenge that may expose it to protein quality control.

The Kir channel subunits share a common structure consisting of two transmembrane domains with an extracellular loop. The extracellular loop of the channel forms a potassium selective pore, which is mediated by the sequence GYG (Figure 3). Mutating this sequence decreases or eliminates potassium selectivity and permeability (Heginbotham et al., 1994). For example, mutating the sequence to AAA completely eliminates channel activity even though expression levels remain similar (McLerie and Lopatin, 2003; Tinker et al., 1996). While the mutation is in the selectivity filter, the intracellular localization of this mutant has not been examined. Finally, the two intracellular termini contain signaling information responsible for directing the intracellular localization of the channels as well as sites for other proteins to modulate channel activity (see section 1.3.2).

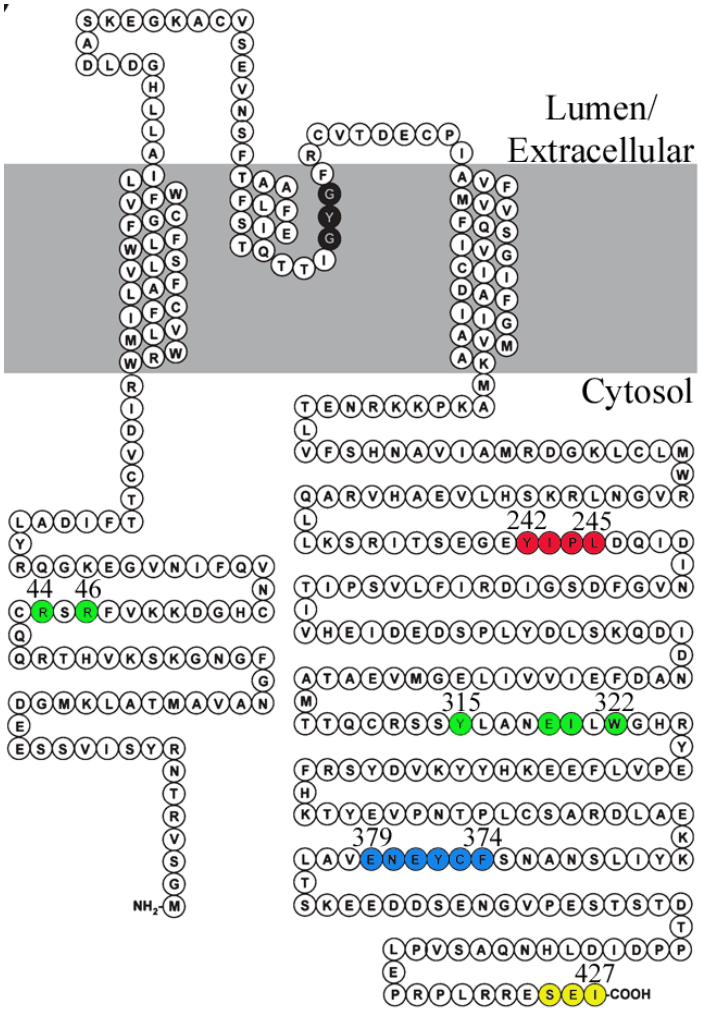


Figure 3: Kir2.1 topology

Kir2.1 contains two transmembrane domains that traverse the membrane (gray) as well as a segment that partially inserts into the membrane. The diagram of Kir2.1 highlights residues that modulate its function/localization with black circles encoding the potassium selectivity filter within the extracellular loop, green circles encoding a Golgi export signal, blue circles encoding an ER export signal, yellow circles encoding a PDZ domain binding site, and the red circles representing a tyrosine endocytic motif (YXXΦ) (adapted from de Boer, et al., 2010)

1.3.2 Kir2.x subfamily

While Kir1, Kir4, and Kir5 are expressed in the kidney, the Kir2 subfamily is primarily expressed in brain, cardiac, smooth, and skeletal muscles (de Boer et al., 2010; Hibino et al., 2010). This subfamily consists of five members: Kir2.1, 2.2, 2.3, 2.4 and 2.6. Kir2.x channels are constitutively active and strong inward rectifiers, although their conductivity and rectification are not equal. Kir2.1, 2.2, and 2.6 homotetramers conduct potassium 2-3 times better than Kir2.3 and 2.4. However Kir2.4 is the strongest inward rectifier while Kir2.1 is the weakest (de Boer et al., 2010). Kir2.1 is the best studied protein in this subfamily and thus represents a strong candidate to explore protein quality control in the secretory pathway.

1.3.2.1 Kir2.1

Kir2.1 was one of the first Kir channels to be cloned and characterized (Kubo et al., 1993). It is responsible for repolarization of the ventricle in cardiac cells. Gain of function mutations in Kir2.1 lead to short QT syndrome, which can cause arrhythmia and sudden death (Priori et al., 2005). In contrast, loss of function mutations cause Andersen-Tawil syndrome in which the QT interval lengthens and causes arrhythmia, and due to its function in other tissues, patients with Andersen-Tawil syndrome also experience periodic paralysis and have developmental abnormalities (Hattori et al., 2012; Plaster et al., 2001). These diseases illustrate the importance of Kir2.1 function in the heart, and as a result, Kir2.1 is being targeted as a biological pacemaker (Hu et al., 2012).

Kir2.1 function in neurons is also medically relevant. Kir2.1 loss of function in medium spiny neurons leads to increased hyperexcitabilty and decreased plasticity, symptoms associated

with Schizophrenia and Parkinson's disease (Cazorla et al., 2012). In support of a role for Kir2.1 in Parkinson's disease, peripheral blood lymphocytes from patients with Parkinson's disease had decreased Kir2.x proteins compared to healthy individuals (Gui et al., 2011). Kir2.1 involvement in maintaining neuronal membrane potentials has led it to be targeted for gene-based neuromodulation (Boulis et al., 2013). Thus, Kir2.1 plasma membrane residence must be tightly regulated.

Kir channels are synthesized in the ER and likely assemble there. Thus, the ER represents the first point of protein quality control and regulation of Kir2.1. If the channel folds and successfully avoids ERAD, ER exit signals in these channels such as FCYENE allow Kir2.1 to traffic to the Golgi, where it is potentially monitored by Golgi protein quality control (Figure 4) (Ma et al., 2001; Stockklausner et al., 2001). Trafficking to the plasma membrane requires a Golgi export signal consisting of a bipartite signal in the N and C termini of Kir2.1 (Ma et al., 2011; Stockklausner and Klocker, 2003). Kir channels then traffic to the plasma membrane where they function and are exposed to a third protein quality control mechanism, plasma membrane protein quality control. Kir2.1 plasma membrane residence is maintained in part by association with proteins such as filamin-A (Pierri et al., 2003).

Kir2.1 interacts with additional plasma membrane proteins including PDZ-domain containing proteins (Figure 3). These interactions lead to the formation of macromolecular complexes that regulate its function (Leonoudakis et al., 2004; Leyland and Dart, 2004; Nehring et al., 2000). For example, the PDZ-domain containing protein, SAP97, interacts with Kir2.1 and forms a macromolecular complex with β-arrestin and protein kinase A (Vaidyanathan et al., 2010). Plasma membrane internalization of Kir2.1 is regulated by an AMP-activated protein kinase mechanism that leads to the phosphorylation of the E3 ubiquitin ligase Nedd4-2. This in

turn activates the ligase, leading to the ubiquitination and subsequent internalization of the channel (Alesutan et al., 2011).

Kir2.1 endocytosis requires a tyrosine based motif in the Kir2.1 C-terminus (Tong et al., 2001). Endocytosis ultimately leads to degradation in the lysosome. For example, lysosomal inhibition with chloroquine, leupeptin, or NH_4Cl increases Kir2.1 protein levels and increased current (Jansen et al., 2008; Nalos et al., 2011). Kir channel regulation may occur at multiple places within the cell and while the determinants in the Kir2.1 sequence and some of the cellular components responsible for its localization have been identified, Kir2.1 protein quality control factors in both the early and late secretory pathway remain undefined.

1.3.2.2 Kir2.1 in yeast

As described above, Kir2.1 has been expressed in yeast, and yeast possess two transporters, Trk1 and Trk2, that are important for maintaining appropriate intracellular potassium levels when grown in low potassium environments. Although both transporters have been shown to have high affinity for potassium, Trk1 is expressed to a greater degree than Trk2 under normal conditions (Arino et al., 2010). Growth on low potassium is rescued by the expression of this channel. The initial system expressed Kir2.1 from either the constitutive *PGK* promoter or the inducible *GAL* promoter (Tang et al., 1995b). The consequence of Kir2.1 expression was further examined in a system that also lacked a potassium efflux system and it was found that these strains were more sensitive to high potassium when Kir2.1 was expressed (Kolacna et al., 2005). This second system was then used to characterize functional elements within the transmembrane domains by examining a Kir2.1 mutant library for growth on low potassium in *trk1Δ trk2Δ* yeast (Minor et al., 1999). Other screens for increased growth of the *trk1Δ trk2Δ* strain identified a Kir2.1 specific inhibitor as well as a mammalian cDNA encoding

the Kir2.1 trafficking factor, TRAK2 (Grishin et al., 2006; Zaks-Makhina et al., 2004). Together, thse data demonstrate yeast's utility as an established model organism for examining Kir2.1 function, trafficking, and structure.

1.4 PROTEIN QUALITY CONTROL

As discussed briefly in the introduction to this chapter, approximately one-third of all translated proteins traverse the secretory pathway in eukaryotes. Many of these proteins may be misfolded, which can lead to cell toxicity. To prevent cell toxicity, there are numerous cellular mechanisms to detect and degrade aberrant proteins including ERAD, Golgi quality control, and plasma membrane quality control.

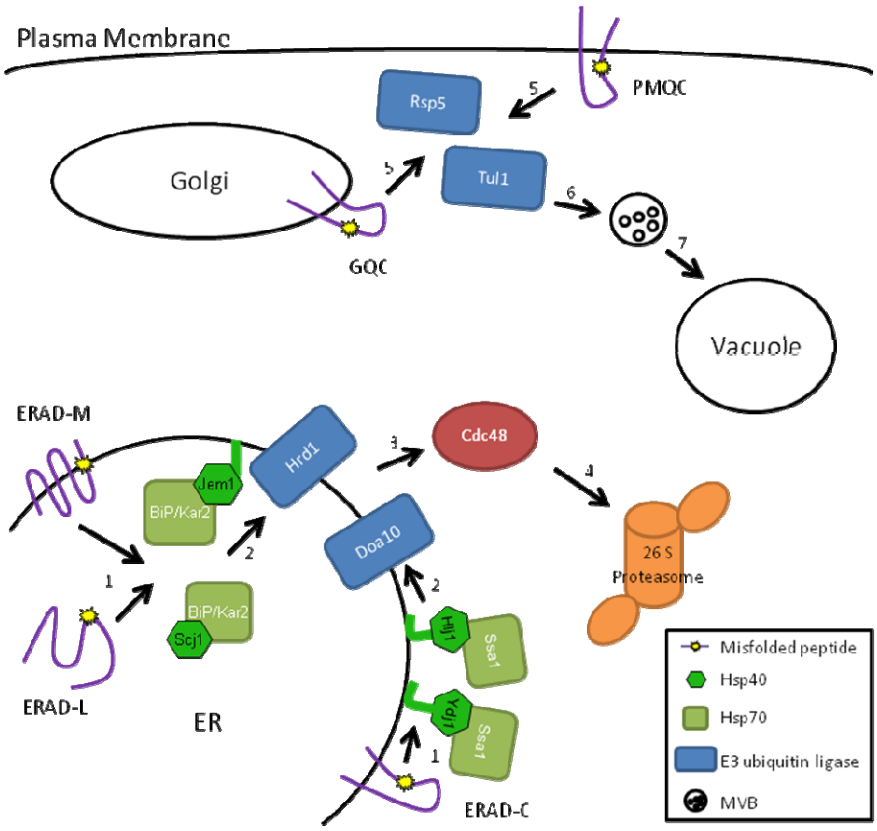


Figure 4: Protein quality control in the secretory pathway

In yeast, misfolded proteins in the ER are targeted by ERAD-C, ERAD-L, or ERAD-M depending on the site of the lesion (yellows star). 1) ERAD substrates are recognized by chaperones (green). 2) Chaperones facilitate delivery to E3 ubiquitin ligases (blue) where they are ubiquitinated. 3) Cdc48 aids in the extraction of substrates. 4) Cdc48 delivers substrates to the 26S proteasome. Misfolded proteins at the Golgi and plasma membrane are targeted for degradation by Golgi quality control (GQC) and plasma membrane quality control (PMQC), respectively. 5) Misfolded substrates are ubiquitinated by E3 ubiquitin ligases. 6) Substrates are delivered into multivesicular bodies (MVBs) (see section 1.5). 7) In turn, MVBs are delivered to the vacuole so that enclosed proteins can be degraded.

1.4.1 Endoplasmic Reticulum Associated Degradation (ERAD)

Protein quality control of secretory proteins begins in the ER with ERAD. In ERAD, misfolded ER substrates are first recognized as substrates. They are then ubiquitinated and retrotranslocated from the ER and eventually targeted to the proteasome for degradation (Araki and Nagata, 2011; Hebert et al., 2010; Smith et al., 2011; Vembar and Brodsky, 2008). ERAD must be able to recognize a variety of substrates that contain misfolded lesions with the cytoplasm, lumen, and membrane (Figure 4). The ERAD-C (cytoplasm), ERAD-L (lumen), and/or ERAD-M (membrane) pathways are composed of specific components that recognize these substrates (Carvalho et al., 2006; Vashist and Ng, 2004).

1.4.1.1 Recognition by chaperones

ERAD substrates must first be recognized as being misfolded. For a non-glycosylated protein, like Kir2.1, this is accomplished primarily by molecular chaperones such as the heat shock proteins i.e., Hsp70s, Hsp40s (Dudek et al., 2009; Mayer and Bukau, 2005). Members of the Hsp70 chaperones bind to hydrophobic patches of substrates via the substrate binding domain (Blond-Elguindi et al., 1993; Flynn et al., 1991; Wang et al., 1993). They then prevent aggregation and can facilitate folding using energy from ATP hydrolysis via the ATPase domain; ATP rebinding and ADP displacement result in substrate release (McCarty et al., 1995). Yeast Hsp70s have been shown to be involved in the ERAD of multiple substrates (Hill and Cooper, 2000; Plemper et al., 1997; Zhang et al., 2001). In yeast, the ER lumenal Hsp70 associated with ERAD substrates, such as CPY*, is Kar2, which is known as BiP in higher cells (Plemper et al., 1997). Membrane bound ERAD substrates with prominent cytoplasmic domains, such as mutant

forms of Pma1 and Ste6, are primarily recognized by the yeast cytosolic Hsp70, Ssa1 (Han et al., 2007; Nakatsukasa et al., 2008).

Hsp40s are Hsp70 cochaperones that are best characterized for their ability to stimulate ATP hydrolysis, although some bind substrates directly, which may allow Hsp40s to deliver substrates to Hsp70s (Laufen et al., 1999; McCarty et al., 1995; Szabo et al., 1994). In yeast there are four Hsp40s associated with ERAD. Scj1 and Jem1 are the lumenal Hsp40s while Ydj1 and Hlj1 are the cytosolic Hsp40s that are involved in the degradation of ERAD-C substrates (Nishikawa et al., 2001; Youker et al., 2004). If the chaperones are unable to facilitate the folding of the misfolded substrate, the protein is delivered to the ubiquitination machinery.

1.4.1.2 Ubiquitination of ERAD substrates

Substrates are ubiquitinated by specific ER-associated or in some cases cytoplasmic E3 ubiquitin ligases (Claessen et al., 2012). Ubiquitin is a small (76 amino acid) ubiquitously expressed peptide that acts as a signaling molecule and controls a variety of cellular functions including the cell cycle, transcription, internalization, and in the case of ERAD, degradation. Chains of ubiquitin are covalently attached to proteins through the coordination of three enzymes: an E1 activating enzyme, an E2 conjugating enzyme, and an E3 ubiquitin ligase. The E1 activating enzyme forms a thiol-ester bond with ubiquitin. The E1 transfers the ubiquitin to the E2 conjugating enzyme. The E2 conjugating enzyme partners with an E3 ubiquitin ligase to attach ubiquitin to the substrate (Claessen et al., 2012; Goder, 2012; Kostova et al., 2007; Spasser and Brik, 2012).

There are two E1s, approximately 40 E2s and over 1000 E3s in mammals (Claessen et al., 2012). In yeast, there is less complexity with only one E1, approximately 12 E2s and around 100 E3s (Kostova et al., 2007). The large number of E3 ubiquitin ligases provides specificity for

the vast number of substrates that need to be ubiquitinated. Hrd1 and Doa10 are the primary E3 ubiquitin ligases that function in the ERAD of most substrates in yeast (Kostova et al., 2007). Hrd1 is associated primarily with the ubiquitination of ERAD-L and ERAD-M substrates such as HMG-CoA reductase, CPY*, Sec61-2, and Pdr5* (Bordallo et al., 1998; Hampton et al., 1996; Plemper et al., 1998). Conversely, Doa10 is associated with ERAD-C substrates such as Ste6* and Pma1-D378N (Huyer et al., 2004b; Wang and Chang, 2003). Although other E3 ubiquitin ligases such as Rsp5 have been implicated in ERAD under different conditions (Haynes et al., 2002), their role as general modulators of substrate degradation is less clear.

1.4.1.3 Retrotranslocation and degradation

To be ubiquitinated, a substrate must access the enzymatic domain of the E3s, which are located in the cytosol. Further, the proteasome, which is responsible for the degradation of ERAD substrates, resides in the cytosol. Thus, all soluble, lumenal ERAD substrates must cross the ER membrane to the cytosol to be degraded. Recognition and retrotranslocation of polyubiquitinated, misfolded, soluble and integral substrates occurs through a disputed channel and is primarily facilitated by the AAA-ATPase, Cdc48 and two cofactors, Npl4 and Ufd1 (Bays and Hampton, 2002; Meyer et al., 2002; Rabinovich et al., 2002; Stolz et al., 2011; Ye et al., 2001). Cdc48 with the aid of additional cofactors then presents the polyubiquitinated substrate to the 26S proteasome for degradation (Richly et al., 2005).

The proteasome is a large proteolytic complex composed of a 20S core and a 19S regulatory particle (Matyskiela and Martin, 2013; Nickell et al., 2009). The 20S core is composed of two sets of seven member protein rings that contain multiple catalytic activities to cleave proteins into short peptides (Groll et al., 1997; Heinemeyer et al., 1997; Lowe et al., 1995). The 19S regulatory particle is composed of multiple proteins that recognize ubiquitinated

substrates and an AAA+ "unfoldase" that denatures proteins and threads them into the proteolytic core (Aubin-Tam et al.; Martin et al., 2008; Thrower et al., 2000). Given that so many proteins are degraded by the 26S proteasome it is not surprising that multiple diseases are due to proteasome dysfunction and proteasome inhibitors have been used therapeutically (Chitra et al., 2012).

1.4.2 Golgi and plasma membrane quality control

There is also a need for protein quality control at later secretory compartments, because not all misfolded proteins are targeted for ERAD. For example, a mutant form of the plasma membrane ATPase, *pma1-7*, is not targeted to the plasma membrane but to the vacuole after passing through the Golgi (Chang and Fink, 1995). A screen to identify genes that when mutated rescued mutant Pma1 localization identified members of retromer (*VPS29*, *VPS35)*, members of phosphatidylinositol 3-kinase II complex (PI3KII) (*VPS38*, *VPS36*), endocytic targeting to the vacuole (*MVP1*, *BSD2*), vesicle tethering (*VPS8*, *SRO77*), a glucosyl transferase (*ALG8*), and an uncharacterized gene (*VPS13*), as involved in this process (Luo and Chang, 1997). Studies of two more chimeric plasma membrane proteins demonstrated that these Golgi quality control substrates were dependent on Vps10, a receptor necessary for transport of CPY to the vacuole (Holkeri and Makarow, 1998; Hong et al., 1996). Golgi quality control not only targets mutant plasma membrane proteins, but also proteins that escape ERAD because the pathway is saturated or because the substrate cannot be recognized by ERAD (Coughlan et al., 2004; Haynes et al., 2002; Spear and Ng, 2003). While it is unclear how these substrates are recognized, studies in yeast show they are ubiquitinated by either Rsp5 or Tul1 and targeted to the vacuole via the multivesicular body (MVB) pathway (Pizzirusso and Chang, 2004; Reggiori and Pelham, 2002;

Wang et al., 2011). While not as well characterized, a Golgi quality control pathway also exists in mammalian cells (Armstrong et al., 1990; Ashok and Hegde, 2009).

Quality control also exists at the plasma membrane. For example, a mutation in the transmembrane domain of the α factor receptor, Ste2, leads to it being degraded by the vacuole faster than the wild-type protein (Jenness et al., 1997). Degradation is dependent on Vps23 (Li et al., 1999), which is a member of the endosomal sorting complex required for transport (ESCRT) (See section 1.5). In mammalian cells, plasma membrane substrates can be ubiquitinated by a E3 ubiquitin ligase known as CHIP and then transferred to the lysosome for degradation via the multivesicular body pathway (Apaja et al., 2010; Okiyoneda et al., 2010) (see 1.5). Thus, it appears that alternative quality control mechanisms that function beyond the ER utilize similar pathways that are dependent on ESCRT mediated degradation by the vacuole/lysosome (Figure 4).

1.5 ENDOSOMAL SORTING COMPLEX REQUIRED FOR TRANSPORT (ESCRT)

As discussed, quality control at the Golgi and the plasma membrane utilizes the MVB pathway, which requires ESCRT. In addition to forming MVBs, ESCRT functions in exosome secretion (Baietti et al., 2012; Wehman et al., 2011), cell abscission (Guizetti et al., 2011; Samson et al., 2008), and autophagy (Filimonenko et al., 2007; Rusten and Stenmark, 2009). ESCRT is composed of five subcomplexes: ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III and the Vps4-Vta1 complex (Henne et al., 2011). Disruption of ESCRT leads to multiple diseases such as cancer (Li and Cohen, 1996; Xu et al., 2003b) and neurodegenration (Filimonenko et al., 2007; Skibinski et al., 2005). In addition, enveloped viruses make use of the ESCRT machinery to bud

from cells (Garrus et al., 2001). However, its role in the formation of MVBs is best characterized.

MVB formation begins with the recruitment of ESCRT-0. ESCRT-0 is composed of Vps27 and Hse1 (Hrs and STAM1/2 in mammals), which are recruited to endosomes through interactions with phosphatidylinositol 3-phosphate and clathrin (Bilodeau et al., 2002; Katzmann et al., 2003; Raiborg et al., 2001). There, they bind ubiquitinated cargo and cluster at the future site of invagination (Bilodeau et al., 2002; Wollert and Hurley, 2010) (Figure 5A). The ESCRT-I complex, composed of Vps23 (Tsg101 in humans), Vps28, Vps37, and Mvb12, is recruited to this site via an interaction between Vps27 and Vps23 (Chu et al., 2006; Katzmann et al., 2001; Katzmann et al., 2003). At this site, ESCRT-I components, Vps23 and Mvb12, assist in binding ubiquitinated cargo (Shields et al., 2009) (Figure 5B). Next, an interaction between Vps28 and Vps36 recruits ESCRT-II, which also consists of Vps22 and Vps25 (Babst et al., 2002b; Teo et al., 2006). ESCRT-I and ESCRT-II recruitment leads to deformation of the membrane (Wollert and Hurley, 2010) (Figure 5C). ESCRT-II recruits and aids in the assembly of ESCRT-III, which consists of a Vps20-Snf7 subcomplex and a Vps24-Vps2 subcomplex (Babst et al., 2002a). Vps20 interacts with Vps25 of ESCRT-II and this leads to the oligomerization of Snf7 that forms a ring around the cargo (Henne et al., 2012; Teo et al., 2004) (Figure 5D). Snf7 oligomerization is stabilized by Bro1 (Alix in humans), which recruits Doa4 to deubiquitinate the cargo so that ubiquitin can be recycled (Dupre and Haguenauer-Tsapis, 2001; Luhtala and Odorizzi, 2004) (Figure 5E). Next, Vps24 and Vps2 drive invagination of the Snf7 oligomer and ultimately lead to termination of oligomer formation (Henne et al., 2012) (Figure 5F). The AAA-ATPase Vps4 and the activating protein Vta1 are recruited by ESCRT-III to disassemble

the complex so that it can be recycled and scission can occur, thereby completing the formation of an MVB (Babst et al., 1997; Babst et al., 1998) (Figure 5G).

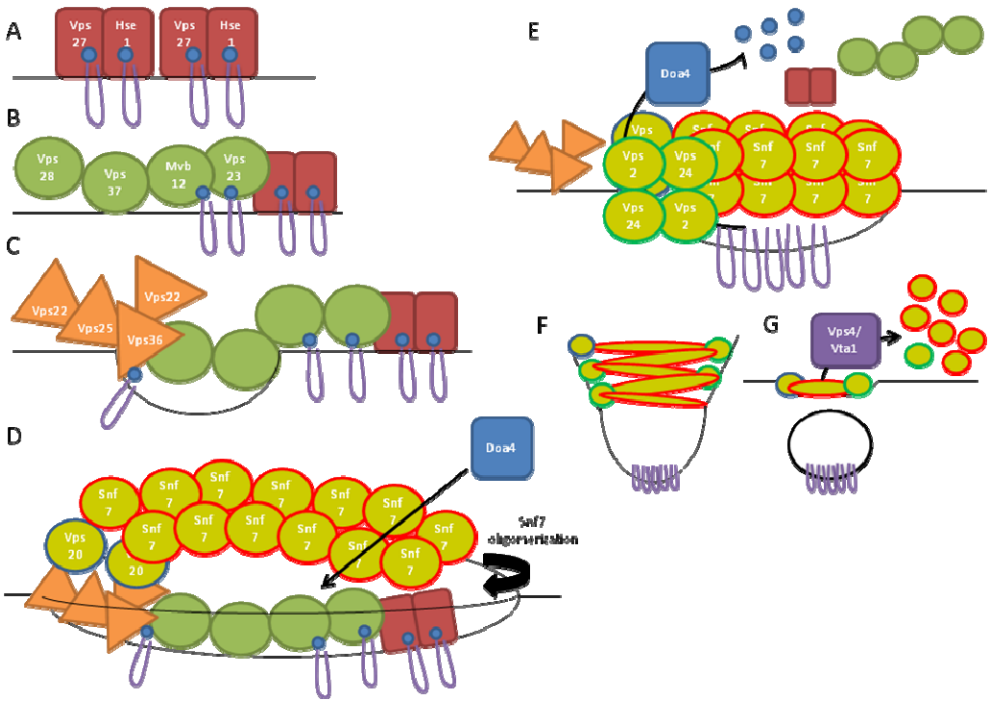


Figure 5: ESCRT functions to produce MVBs

Cargo (purple) marked with ubiquitin (blue circle) is sequestered into an internal vesicle through the actions of ESCRT-0 (red), ESCRT-I (green), ESCRT-II (orange), ESCRT-III (yellow), and the Vps4/Vta1 complex. A) ESCRT-0 sequesters cargo. B) ESCRT-I is recruited and aids in cargo sequestration. C) ESCRT-II is recruited and vesicle invagination is initiated. D) ESCRT-III begins to encircle the cargo and recruits Doa4. E) Vps2 and Vps24 are recruited and Doa4 deubiquitinates the cargo, releasing ESCRT-0, ESCRT-I and ESCRT-II. F) Vps2 and Vps4 drive invagination of the Snf7 oligomer. G) Vps4 and Vta1 aid in scission and disassembly of ESCRT-III.

Overall the coordination of these ESCRT subcomplexes confers the ability to transfer a membrane bound protein to the lumen of the vacuole/lysosome. Thus, this pathway is ideally suited for disposing of membrane proteins, such as Kir2.1, that have trafficked beyond the ER and that may still be partially misfolded.

1.6 THESIS OUTLINE

Protein quality control is an active area of investigation and protein quality control systems have been found in many cellular compartments (Arvan et al., 2002; Chen and Lamb, 2008; Chhangani et al., 2012). The secretory pathway represents a unique area to study protein quality control as a protein may be synthesized in the ER, mature in the Golgi, and function at the plasma membrane. Thus, there is a need for protein quality control at all of these compartments, and not surprisingly such systems have been identified. However, the complete set of factors involved in these systems remains unknown. Moreover, how these systems coordinate with each other is unclear.

In chapter 2, I present my work addressing how the secretory protein, Kir2.1, is monitored by protein quality control at multiple sites within the cell. To accomplish this, I engineered a yeast strain deleted for the potassium transporters, Trk1 and Trk2, whose growth on low potassium depends on the potassium channel Kir2.1 functioning at the cell surface. I then crossed this strain to the yeast deletion collection using synthetic gene array methodology and screened the resulting strains on low potassium media for effectors of Kir2.1 plasma membrane residence. I identified 53 genes (Appendix A) including several members of the ESCRT

complex whose deletion leads to increased growth and thus increased plasma membrane residence of Kir2.1. A collaboration with the Welling lab showed that ESCRT regulates the trafficking of Kir2.1 in HeLa cells demonstrating the utility of yeast to study mammalian protein trafficking and protein quality control. In chapter 3 I discuss my findings with an emphasis on alternative approaches and areas of future study. I also included an appendix that describes my work examining the protein quality control of ENaC.

2.0 A YEAST GENOMIC SCREEN REVEALS ESCRT REGULATES KIR2.1 PLASMA MEMBRANE RESIDENCE

2.1 INTRODUCTION

Protein quality control systems exist throughout the eukaryotic secretory pathway to ensure that toxic misfolded proteins or protein complexes do not accumulate. Quality control systems may also act as a means to regulate protein levels and thus their activities. When protein quality control is defective or fails to recognize an aberrant protein, human diseases may result (Gestwicki and Garza, 2012; Guerriero and Brodsky, 2012).

In the secretory pathway, protein quality control begins in the endoplasmic reticulum (ER). Proteins that fail to pass quality control may be destroyed by a process termed endoplasmic reticulum-associated degradation (ERAD). During ERAD, substrates are first recognized by molecular chaperones and chaperone-like lectins, which target them to the ER-associated ubiquitination machinery (Aebi et al., 2010; Claessen et al.; Hebert and Molinari, 2012). During or after ubiquitination, ERAD substrates are retrotranslocated to the cytoplasm and delivered to and degraded by the 26S proteasome (Mehnert et al., 2010; Smith et al., 2011). However, if the ERAD machinery is overwhelmed, or aberrant proteins are not recognized by the ERAD machinery, the unfolded protein response is induced and in some cases misfolded species may escape the ER and advance to the Golgi. In these circumstances, ERAD substrates may be

returned to the ER and degraded (Haynes et al., 2002; Taxis et al., 2002; Vashist et al., 2001) or a Golgi-resident quality control system can recognize and deliver substrates to the vacuole/ lysosome for degradation via the multivesicular body (MVB) pathway (Chang and Fink, 1995; Coughlan et al., 2004; Hong et al., 1996; Spear and Ng, 2003; Wang and Ng, 2010). Protein quality control mechanisms also exist at the plasma membrane, and in this case selected substrates are endocytosed and targeted to the lysosome (Huotari and Helenius, 2011). For example, the disease-associated ΔF508 mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) is normally an ERAD substrate but is less stable than wild type CFTR when artificially delivered to the plasma membrane (Okiyoneda et al., 2010). A large scale screen indicated that a significant number of factors are required for the disposal of ΔF508 CFTR (Trzcinska-Daneluti et al., 2009). In general, plasma membrane quality control, like Golgi quality control (GQC), appears to use the MVB pathway to route substrates to the vacuole (Apaja et al., 2010; Arvan et al., 2002; Jenness et al., 1997). Together, there are several protein quality control mechanisms in place to ensure that unwanted secreted proteins are destroyed, but it is unclear how these different mechanisms cooperate.

The functions of distinct protein quality control networks must be linked with the machineries that regulate protein localization. Multiple screens have been performed to identify the factors that regulate the localization and trafficking of proteins, and one of the critical trafficking complexes that delivers proteins from the plasma membrane and Golgi to the vacuole/lysosome is the endosomal sorting complex required for transport (ESCRT) (Bankaitis et al., 1986; Bonangelino et al., 2002; Robinson et al., 1988; Rothman et al., 1989). ESCRT functions primarily in the formation of MVBs (MacGurn et al., 2012), which begins with the recognition and initial clustering of ubiquitinated cargo by ESCRT-0 (Bilodeau et al., 2002;

Mayers et al., 2011; Wollert and Hurley, 2010). ESCRT-I and ESCRT-II are then recruited and confine cargo at the site of vesicle formation, which initiates vesicle invagination before ESCRT-III is recruited (Gill et al., 2007; Kostelansky et al., 2007; Teo et al., 2006; Teo et al., 2004; Wollert and Hurley, 2010). Association of ESCRT-III leads to cargo deubiquitination (McCullough et al., 2006) and vesicle maturation (Wollert and Hurley, 2010; Wollert et al., 2009). The Vps4-Vta1 complex then aids in disassembly of the ESCRT complex to complete vesicle formation (Azmi et al., 2008; Babst et al., 1998). In addition to Golgi and plasma membrane quality control, ESCRT is also required for cell abscission and viral budding in metazoans (Carlton et al., 2008; Carlton and Martin-Serrano, 2009; Chen and Lamb, 2008; Xie et al., 1998) and autophagy (Rusten and Stenmark, 2009). Defects in ESCRT function may lead to human diseases, including cancer and neurodegeneration (Filimonenko et al., 2007; Li and Cohen, 1996; Saksena and Emr, 2009; Skibinski et al., 2005; Xu et al., 2003b).

In addition to serving as a tool to identify components that comprise the trafficking machinery, yeast systems have also been co-opted to examine the protein quality control and trafficking of specific mammalian proteins (Franssens et al., 2009; Kolb et al., 2011; Winderickx et al., 2008). In some cases these analyses were facilitated by employing specific genomic tools such as synthetic gene array (SGA) methodology (Tong and Boone, 2006). For example, a screen was performed in which the inward rectifying potassium channel 3.2 (Kir3.2) was mutated so that it was sodium-selective, and strains expressing the protein were crossed to a subset of the deletion collection (Haass et al., 2007). The resulting haploids were screened on medium containing high sodium and seven genes associated with COPII-dependent transport and lipid biogenesis were identified that when mutated reduced sodium-mediated toxicity. These hits were confirmed when a potassium-selective Kir3.2 construct was expressed that led to increased

growth in a yeast strain lacking the Trk1 and Trk2 potassium transporters (Haass et al., 2007). Trk1 and Trk2 are the main route for potassium influx into yeast in low extracellular potassium, and when they are deleted yeast are only able to grow in the absence of high potassium if an exogenous potassium channel is expressed (Nakamura and Gaber, 1998).

The Kir2 potassium channels form a tetramer and function primarily in heart, skeletal muscle, and neurons, and consists of five family members, Kir2.1-2.4, and 2.6 (de Boer et al., 2010; Hibino et al., 2010). Gain of function mutations in Kir2.1 lead to short QT syndrome, which can cause arrhythmia and sudden death (Priori et al., 2005). In contrast, loss of function mutations in Kir2.1 result in Anderson-Tawil syndrome (Plaster et al., 2001). Kir2.1 function in cardiac tissue has led to efforts to target its levels as a biological pacemaker (Hu et al., 2012). Kir2.1 loss of function in medium spiny neurons also leads to increased hyperexcitabilty and decreased plasticity, symptoms associated with schizophrenia and Parkinson's disease (Cazorla et al., 2012). These cases illustrate the importance of maintaining proper Kir2.1 levels at the plasma membrane. However, the factors necessary for Kir2.1 biogenesis, trafficking, and stability have not been explored systematically.

To this end, I utilized a new yeast Kir2.1 expression system. Like many complex membrane proteins (Jensen et al., 1995; Needham et al., 2011; Tamarappoo et al., 1999; Valentijn et al., 1998; VanSlyke et al., 2000; Ward et al., 1995), the Brodsky lab found that the majority of Kir2.1 is ER localized and targeted for ERAD. Specifically, Kir2.1 degradation was dependent on the AAA-ATPase, Cdc48, the ER-associated E3 ubiquitin ligases, Hrd1 and Doa10, and the cytoplasmic Hsp70 chaperone, Ssa1. Because Kir2.1 supports the growth of *trk1Δ trk2Δ* on low potassium (Nakamura and Gaber, 1998; Tang et al., 1995b; Zaks-Makhina et al., 2004), this system also provided us with the unique opportunity to identify the spectrum of

protein quality control and trafficking factors necessary for the maintenance of active Kir2.1 at the plasma membrane. Interestingly, ERAD-requiring factors were absent from the yeast mutants that supported growth on low potassium medium, suggesting that the level of functional Kir2.1 is not primarily regulated by ERAD. In contrast, most of the ESCRT complex components were isolated from our screen. These data indicate that later steps during secretory protein quality control are required for the maintenance of Kir2.1 at the plasma membrane. Consistent with this hypothesis, ESCRT also regulated Kir2.1 residence at the plasma membrane in human cultured cells. Together, these data provide the first genomic analysis of how a single protein can be subject to multiple quality control pathways, and demonstrate that only specific quality control pathways may need to be modulated to correct unique disease-associated membrane proteins.

2.2 MATERIALS AND METHODS

2.2.1 Yeast strains and growth conditions

Yeast strains were propagated at 26°C, and standard methods for growth, media preparation, and transformation were used unless indicated otherwise (Adams, 1998). Yeast with plasmids containing constitutive promoters (GPD or TEF) were grown in selective medium and harvested at the optical density (OD) indicated in each experiment. Yeast harboring plasmids containing the *MET25* promoter were maintained on medium containing 0.5mM methionine to inhibit synthesis of the desired protein. To induce Kir2.1 expression, cells were grown to log phase in selective medium supplemented with methionine, collected by centrifugation and washed in

sterile water, and then resuspended in selective medium lacking methionine for 1-2 h. After protein induction, cells were used as described for each experiment. For low potassium growth assays and the SGA screen, 20mM MES and the indicated concentration of potassium were added. Medium was also prepared at the desired pH with Tris-HCl. A complete list of the strains used in this study is presented in Table 2.

Table 2: Yeast strains

Strain	Relevant Genotype	Source
BY4742	*MATα his3, leu2, ura3*	Invitrogen
pdr5Δ	*MATα, his3, leu2, ura3, pdr5::KANMX*	Invitrogen
HRD1/DOA10	*MATα, ade2, his3, leu2, ura3, trp1*	(Pagant et al., 2007)
hrd1Δ	*MATα, ade2, his3, leu2, ura3, trp1, hrd1::KANMX*	(Pagant et al., 2007)
doa10Δ	*MATα, ade2, his3, leu2, ura3, trp1, doa10::KANMX*	(Pagant et al., 2007)
hrd1Δ doa10Δ	*MATα, ade2, his3, leu2, ura3, trp1,hrd1, doa10::KANMX*	(Pagant et al., 2007)
pep4Δ	*MATα, his3, leu2, ura3, pep4::KANMX*	Invitrogen
pep4Δ pdr5Δ	*MATα, his3, leu2, ura3, pep4::KANMX, pdr5::KANMX*	This Lab
SSA1	*MATα, his3-11,15, leu2-3,112, ura3-52, trp1-Δ1, lys2, ssa2-1(LEU), ssa3-1(TRP1), ssa4-2(LYS2),*	(Becker et al., 1996)
ssa1-45	*MATα, his3-11,15, leu2-3,112, ura3-52, trp1-Δ1, lys2, ssa1-45, ssa2-1(LEU), ssa3-1(TRP1), ssa4-2(LYS2),*	(Becker et al., 1996)
CDC48	*MATα his3, leu2, ura3*	This Lab
cdc48-2	*MATα his3, leu2, ura, cdc48-2::KANMX*	This Lab
R5421	*ura3-52 his3Δ200 leu2Δ1 trp1Δ1 ade2 trk1::HIS3 trk2::HIS3*	(Nakamura and Gaber, 1998)
SGY1528	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1*	(Haass et al.,

	ura3-1 trk1::HIS3 trk2::TRP1	2007)
Y7029	*can1Δ::STE2pr-HIS3 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	(Tong and Boone, 2007)
Query Strain (YAK01)	*can1Δ::STE2pr-HIS3 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 trk1Δ::URA trk2Δ::NAT*	This Lab
vps23Δ (YAK02)	*can1Δ::STE2pr-HIS3 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 trk1Δ::URA trk2Δ::NAT vps23Δ::KANMX*	This Lab
doa10Δ (YAK03)	*can1Δ::STE2pr-HIS3 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 trk1Δ::URA trk2Δ::NAT doa10Δ::KANMX*	This Lab

For growth assays, 5 mL cultures were grown to log phase in synthetic complete medium lacking leucine (SC-Leu) but containing 100mM KCl at 30°C, and 1 OD_{600} of cells was collected by centrifugation. The yeast were resuspended in 500 µL sterile water. Cells were serially diluted 10 fold and plated using a 32 pin manifold onto SC-Leu medium with 0, 10, 25, or 100 mM KCl added (potassium levels in regaents is estimated to be 7-10 mM (Nakamura and Gaber, 1998)) and were grown at 30° for 2d. Plates were imaged using an EPSON Perfection 3490 Photo Scanner. To assay for protein expression, an equal number of cells was taken from the plates, and total protein was precipitated with 10% trichloroacetic acid and resolved by SDS-PAGE before western blot analysis (Zhang et al., 2001). Kir2.1 was detected using an anti-HA-horseradish peroxidase (HRP)–conjugated antibody and blots were probed with anti-G6PD antiserum to provide a loading control. The G6PD primary antibody was decorated with donkey HRP-conjugated anti–rabbit immunoglobulin G (IgG) secondary antibody. The Supersignal Chemiluminescent Substrate (Pierce, Rockford, IL) was used to develop the blots, and the signals were quantified using a Kodak 440CF Image Station and the associated Kodak 1D software (Eastman Kodak, Rochester, NY).

Strain YAK01 (see Table 2) was constructed from Y7029 by homologous recombination (Brachmann et al., 1998). The NAT cassette was PCR amplified using primers oAK01, oAK02 (Table 3) that also contained homology to the *TRK2* locus and the PCR product was used to delete *TRK2* in Y7029. The *URA3* cassette from pRS426 (Mumberg et al., 1995) was PCR amplified using primers oAK05, oAK06 (Table 3) that contained homology to the *TRK1* locus. The PCR product was used to delete *TRK1* in Y7029. YAK02 and YAK03 were constructed from AK01 by mating this strain with a *vps23Δ* and a *doa10Δ* mutant from the yeast deletion collection (Invitrogen), respectively. All constructed strains were confirmed by growth on

selective medium and by PCR using specific confirmation primers (Table 3). *CDC48* and *cdc48-2* were constructed by mating *cdc48-2* (Li et al., 2011) to *ybr074Δ* (Invitrogen), and the identity of the desired genotype was confirmed by plating on medium containing G418 (Research Products International) and sequencing following PCR amplification of the locus).

Table 3: Oligonucleotide primers

Primer	use	Sequence
oAK01	Deletion of Trk2	5'-tgtactattcaccgacgataagaggctgtaagaaccactccggatccccgggttaattaa-3'
oAK02	Deletion of Trk2	5'-acgttggctcttatgtaggtaaagaggggtaaacttgattgaattcgagctcgtttaaac-3'
oAK03	Confirm trk2Δ	5'-ctcctgtgaagaataggatgaga-3'
oAK04	Confirm trk2Δ	5'-gctgttgaatcgtagtggtac-3'
oAK05	Deletion of trk1	5'-cattcctatccatttttacttaaagttattacctttttttgataactaacaagattgtactgagagtgca-3'
oAK06	Deletion of trk1	5'-ttcttaaatattgagtacgaaaacctatttctaaagaatgagtatatatgctatgaccatgattacgcca-3'
oAK07	Confirm trk1Δ	5'-cactgattccacacttgtattgcc-3'
oAK08	Confirm trk1Δ	5'-ggacatcagaaatgtacgtaggca-3'
oAK09	Confirm vps23Δ	5'-acatgaacaccatcagtagttcctt-3'
oAK10	Confirm vps23Δ	5'-gtctatctgtgcgtttagcgagt-3'
kanC	Internal KanMx to confirm deletions	5'-tgattttgatgacgagcgtaat-3'
oAK11	mutant construction pcr-a	5'-tagtggatcccccgggctgcaggaatt-3'
oAK12	Kir2.1Δ314-315 construction pcr-a	5'-gaatttcattggccagactccggcattgagttg-3'
oAK13	Kir2.1Δ314-315 construction pcr-b	5'-caactcaatgccggagtctggccaatgaaattc-3'
oAK14	mutant construction pcr-b	5'-gctatagttcgaatagctatggcagctgg-3'
oAK15	GYG-AAA construction pcr-a	5'-tccatcgagacccagacaaccattgccgctgctttcaggtgtgtgacagacgagtgccc-3'
oAK16	GYG-AAA construction pcr-b	5'-gggcactcgtctgtcacacacctgaaagcagcggcaatggttgtctgggtctcgatgga-3'
Myc-Fwd	Amplification of Myc-Kir2.1-HA	5'-gagcagaagctgatatctg-3'
Kir2-REV	Amplification of Myc-Kir2.1-HA	5'-atctccgattctcgcc-3'

2.2.2 Plasmid construction

Mouse Kir2.1 in pcDNA3.1 was modified with an internal HA tag that was positioned so that it faced the extracellular space (Ma et al., 2001). To create a yeast Kir2.1-HA expression construct with a C-terminal myc tag (myc-Kir2.1-HA), the Kir2.1 coding sequence within the pcDNA3.1 vector was PCR amplified using the PCR Master Mix reagent (Fermentas). The amplified Kir2.1 sequence was purified using the Purelink PCR purification kit (Invitrogen) and ligated into the pGEM-T easy vector (Promega). The pGEM-T-Kir2.1 plasmids were sequenced to identify clones that contained the correct Kir2.1 insert. The insert was removed from pGEM-T using EcoRI, the fragment was gel purified using PureLink^{TM} Quick Gel Extraction Kit (Invitrogen) and the isolated species was ligated into the EcoRI site of yeast plasmids containing either constitutive (*TEF*, *GPD*) or regulated (*MET25*) promoters (Mumberg et al., 1994; Mumberg et al., 1995). The myc- Kir2.1-HA insert was then cloned into digested pRS vectors containing different promoters and auxotrophic selections (Mumberg et al., 1994; Mumberg et al., 1995) using SmaI and XhoI. The cut vector was treated with antarctic phosphatase (New England Bioloabs) and the digested vectors were run on a 1% agarose gel and purified with PureLink^{TM} Quick Gel Extraction Kit (Invitrogen). The cut vectors and inserts were finally ligated using T4 DNA Ligase (Fermentas). Kir2.1-AAA and Kir2.1Δ314-315 were made by two stage PCR mutagenesis (Vallejo et al., 1994). The Kir2.1 mutated cassettes were inserted into the PRS415TEF vectors as described above. All isolated inserts were subject to DNA sequence analysis.

2.2.3 Genetic screening conditions

The query strain (YAK01) was mated to the *MAT*a strain deletion collection (Invitrogen) as described (Tong and Boone, 2006). In brief, the query strain was plated onto SC-Leu plates using a 96 pin manifold (Aladin Enterprises Inc., Birsbane, CA). Strains in the deletion collection were then plated over the query strain and plates were incubated at 30°C for 1 d. The cells were then plated onto SC-Leu containing 200 mg/L G418 (Research Products International), 100 mg/L clonNAT (Werner Bioagents, Germany), and 100 mM KCl to select for diploids and to maintain a potassium-sensitive phenotype and were incubated at 30°C for 2 d. The manifold was sequentially washed with water, 10% bleach, 2 times in water, in ethanol and then flamed between each plate. Next, the diploid cells were plated onto sporulation medium and kept at room temperature for 5 d. Finally, the spores were plated on diploid selection media lacking histidine, arginine, and uracil and containing 50 mg/L canavanine (Sigma, St. Louis, MO) and then were incubated at 30°C for 2d. This step was repeated 2 additional times with 1 d incubations to select against any remaining diploids. The resulting haploid strains, each expressing Kir2.1, but deleted for *TRK1*, *TRK2*, and one of ~5000 genes in the collection, were screened on SC -Leu medium lacking potassium and were examined after 2 d at 30°C.

Colonies that grew significantly better than the average colony size on the plate were verified by growth in liquid SC-Leu media containing 100 mM KCl at 30°C in a 96 well plate. A total of 5 µL of cells were then pipetted into 195 µL of SC-Leu medium lacking KCl and grown at 30°C with shaking. The OD_{600} was measured every 1-2 h with a Thermo Scientific Multiskan Go plate reader to obtain the doubling times. All experiments were performed in triplicate.

2.2.4 Indirect immunofluorescence in yeast

Yeast cells expressing Kir2.1 were grown to an OD_{600} of 0.5 - 0.8 and fixed with 3.7% formaldehyde for 1 h at 30°C. The cells were then collected by centrifugation and washed with solution A (2 M sorbitol, 0.5M KPO4 pH 7) and resuspended in 500 µl solution A supplemented with 60 ng/ml 100T zymolyase (MP Biomedicals, Solon, OH) and 25 mM 2-mercaptoethanol and were incubated at 37°C for 30 min. The spheroplasts were next pelleted at 3000 rpm for 3 min, washed with solution A, and resuspended in 800 µl of solution A. The cell suspension (30 µl) was added to a microscope slide well (pretreated with 1 mg/ml polylysine) and incubated at room temperature for 30 min. The cell suspension was aspirated and the wells were washed once with PBS/0.1% bovine serum albumin (BSA) and twice with PBS/0.1% BSA/0.1% NP40, and then incubated with the following primary antibodies at 4°C: anti-HA at 1:250 dilution, and anti-Kar2 at a 1:500 dilution. The slides were next washed once with PBS/0.1% BSA, once with PBS/0.1%BSA/0.1% NP40, and once with PBS/0.1% BSA, and were incubated with the appropriate secondary antibodies (Alexa Fluor 568 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse) in PBS/0.1% BSA for 1 h at room temperature. The slides were then washed as above, and coverslips were mounted using ProLong Gold Antifade mounting media (Invitrogen). Images were captured on an Olympus BX60 microscope (Olympus, Tokyo, Japan) fitted with a Hamamatsu C4742–95 digital camera (Hamamatsu, Bridgewater, NJ), and using QED Imaging software (Media Cybernetics, Silver Spring, MD).

2.2.5 Biochemical assays

Kir2.1 degradation was assayed by cycloheximide chase analysis as described (Buck et al., 2010). In brief, cells were grown in liquid selective medium with shaking to log phase (OD_{600} 0.4-1.2), and 100 μM cycloheximide (Sigma, St. Louis, MO) was added to stop protein translation. The yeast were incubated at 30° in a shaking water bath, and temperature sensitive mutants were incubated at 37°C for 10 min prior to performing the chase at 37°C. The *cdc48-2* temperature sensitive strain and the isogenic wild-type were incubated at 39°C for 2 h prior to the start of the chase, and the incubation was continued at 39°C. A 1 mL aliquot of the culture was collected at the indicated time points and pelleted cells were washed with ice cold water containing protease inhibitors, flash-frozen in liquid nitrogen and stored at -80°C. Total protein was precipitated from samples as described (Zhang et al., 2001) and immediately resolved by SDS–PAGE before western blot analysis, as described above.

To measure the relative amount of Kir2.1 ubiquitination, cells harboring the pRS426MET25-Kir2.1 plasmid were grown to an OD_{600} of 1.0 in 50 ml cultures of selective medium containing 0.5 mM methionine. The yeast were collected by centrifugation, washed in sterile water, and resuspended in the same medium lacking methionine for 2 h. After induction, the cells were collected by centrifugation and quick frozen in liquid nitrogen and stored at -80 C until use. Immunoprecipitations were preformed essentially as described (Ahner et al., 2007). In brief, cells were broken by glass bead lysis, and membranes were collected by centrifugation. The membranes were then treated with SDS buffer to liberate Kir2.1 and after dilution into a TritonX-100-containing buffer an anti-HA antibody conjugated to agarose beads (Pierce) was added, and the slurry was incubated overnight with rocking at 4 °C. The beads were washed, and then SDS sample buffer was used to liberate the bound proteins. The released proteins were

subjected to SDS-PAGE and western blot analysis to detect total Kir2.1 and the ubiquitinated Kir2.1 fraction.

The intercellular residence of Kir2.1 was determined by sedimentation in a sucrose gradient essentially as described (Sullivan et al., 2003). A 40 ml culture was grown to an OD_{600} of 0.8 and the cells were harvested by centrifugation, resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% sucrose, and then disrupted by agitation with glass beads. The cell lysates were cleared of debris by low speed centrifugation and the resulting lysate (300 µl) was layered on the top of a 1mL 30-70% sucrose gradient and centrifuged at 100,000x*g* in a Beckman SW41 rotor for 14 h at 4^{o}C. Fractions were collected by pippeting from the top of the tube and proteins were analyzed by SDS-PAGE and western blotting for Kir2.1 with anti-HA antibody or antisera against the ER (Kar2/BiP) and plasma membrane (Pma1) resident proteins.

2.2.6 Analysis of Kir2.1 in HeLa cells

HeLa cells were cultured in DMEM with 10% FBS, 100U/mL penicillin/streptomycin and 2 mM glutamine at 37°C, 5% CO_2. XtremeGENE9 transfection reagent (Roche) was used as per the manufacturer's guidelines to transfect mouse Kir2.1 in pcDNA3.1 (pcDNA3.1-HA-Kir2.1). To knock-down the expression of ESCRT members, HeLa cells plated in 6-well dishes were co-transfected with 1µg pcDNA3.1-HA-Kir2.1 and 2 µg siRNA using XtremeGENE siRNA transfection reagent. The siRNAs for human TSG101 (CCAAAUACUUCCUACAUGC), human HRS (HGS) (GAACCCACACGUCGCCUUG), and a scramble control- ON-TARGETplus non-targeting siRNA #1 were obtained from Invitrogen. After 54 h 100 µL leupeptin was added. After another 18 h cells were washed with PBS and collected in 1% Triton in PBS with Protease Inhibitor Cocktail (Sigma, P8340). Lysates were rotated for 1 h at

4°C, followed by centrifugation at 13,000RPM for 5 min at 4°C. Supernatants were incubated with SDS loading buffer for 30 min at room temperature.

To determine the extent to which Kir2.1 accumulates in MVBs in HeLa cells, Kir2.1-HA was co-localized with the MVB marker, CD63 (Escola et al., 1998), using a rabbit monoclonal HA- antibody and a mouse monoclonal CD63 antibody. For these studies, cells were grown on glass coverslips and then fixed (4% paraformaldehyde, 30 min, 5°C), permeabilized with 0.1% Triton X-100 (10 min, 5°C) and blocked in 5% FBS (30 min, 5°C). The cells were then incubated with the primary antibodies (1:100, 2 h, 5°C), washed, and incubated with anti-mouse and anti-rabbit AlexaFluor conjugated secondary antibodies (1:250, 1 h at room temperature). After washing, the cells were mounted in VectaShield mounting media containing the nuclear marker, DAPI. The signals corresponding to each protein were visualized with a Zeiss 510 confocal microscope using 63X oil immersion lens (NA = 1.4), and images were processed using Volocity image analysis software (PerkinElmer).

For immunoblot analysis, cells were washed with PBS and collected in 1%Triton X-100 in PBS with Protease Inhibitor Cocktail (Sigma, P8340) and then incubated with rotation for 1 h at 4°C and then centrifuged at high speed (13,000RPM, 5 min 4°C) to pellet insoluble material. Supernatant protein concentration was determined by BCA Protein Assay Kit (Pierce) and proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with the appropriate primary and HRP-conjugated secondary antibodies. SuperSignal West Pico Chemiluminescent Substrate was used to detect the signal from the HRP –conjugated antibody, and the chemiluminescent signal was visualized by fluorography. Densitometric measurements were made in the linear range using NIH software, ImageJ.

To monitor Kir2.1 stability, 50 µg/mL cycloheximide (Sigma, St. Louis, MO) in the presence or absence of 15 µg/mL MG132 (Peptides International) were added to cells expressing Kir2.1-HA to stop protein translation and inhibit the proteasome, respectively. The cells were incubated at 37°C and were harvested with 100 µL lysis buffer (50mM Tris pH7.2, 1% Triton X-100, 150 mM NaCl, cOmplete Mini protease inhibitor cocktail (Roche Diagnostics)), and the protein concentration was measured by BCA (Thermo Scientific). Equal amounts were resolved by SDS–PAGE before western blot analysis.

2.2.7 Statistical Analysis

To analyze experiments performed in HeLa Cells (Figure 15), Statistical analysis was performed using GraphPad Prism. Statistical significance was determined by one-way ANOVA and TuKeys post hoc test in G, and two-way ANOVA and Bonferroni post tests in H.

2.2.8 Antibodies

The following primary antibodies were used for studies in yeast: horseradish peroxidase (HRP)-conjugated rat monoclonal anti-HA high affinity (3F10, Roche Applied Science), anti-HA mouse monoclonal (Roche, Indianapolis, IN), G6PD rabbit polyclonal antibody (Sigma-Aldrich, St. Louis, MO), a ubiquitin (P4D1) mouse monoclonal antibody from Santa Cruz (sc-8017), a polyclonal rabbit anti-Kar2 (Brodsky and Schekman, 1993). A polyclonal rabbit anti-Pma1 was a gift from Amy Chang (University of Michigan, Ann Arbor, MI). The following secondary antibodies were used in yeast: HRP-conjugated goat anti-mouse and goat anti-rabbit antibodies (Jackson ImmunoResearch), Alexa Fluor 568 goat anti-rabbit and Alexa Fluor 488 goat anti-

mouse (Invitrogen, Carlsbad, CA). The following antibodies were used for studies in HeLa cells: HA mouse monoclonal antibody (Covance), HA rabbit monoclonal antibody (Cell Signaling), CD63 mouse monoclonal antibody (Invitrogen), TSG101 mouse monoclonal antibody (GeneTex), HRS (HGS) mouse monoclonal antibody (Abcam), and VPS35 goat polyclonal antibody (Abcam). The following secondaries were used for immunofluorescence studies in HeLa cells: anti-mouse and anti-rabbit AlexaFluor conjugated secondary antibodies (Molecular Probes)

2.3 RESULTS

2.3.1 Kir2.1 is an ERAD substrate

The Kir2.1 potassium channel must traverse the secretory pathway and most likely encounters multiple levels of protein quality control. For example, a disease-causing mutation in the channel, Δ314-315, prevents the trafficking of the channel from the Golgi apparatus to the plasma membrane by virtue of its inability to associate with AP-1 (Ma et al., 2011). To examine whether Kir2.1 is also subject to ER quality control, a new yeast expression system was established, which permits a rapid means to analyze the ERAD requirements for a given substrate. To this end, a double tagged (HA-Kir2.1-Myc) version of Kir2.1 was expressed under the control of the constitutive GPD promoter. Analysis of Kir2.1 localization by indirect immunofluorescence primarily showed a perinuclear staining that colocalized with the ER resident chaperone, Kar2 (BiP) (Figure 6A). Sucrose gradient centrifugation analysis indicated that Kir2.1 was also mostly present in the same fractions as Kar2 (Fig. 5B lanes 1-6). However,

a fraction (<5%) of Kir2.1 comigrated with the plasma membrane protein Pma1 (lanes 9-11). These data suggest that while the majority of Kir2.1 is localized in the ER, a small pool advances to the plasma membrane in yeast. This is in contrast to what is observed in mammalian cells in which the majority of Kir2.1 traffics beyond the ER (Stockklausner et al., 2001). This may suggest that yeast lack Kir2.1 assembly or anterograde trafficking factors important for its biogenesis.

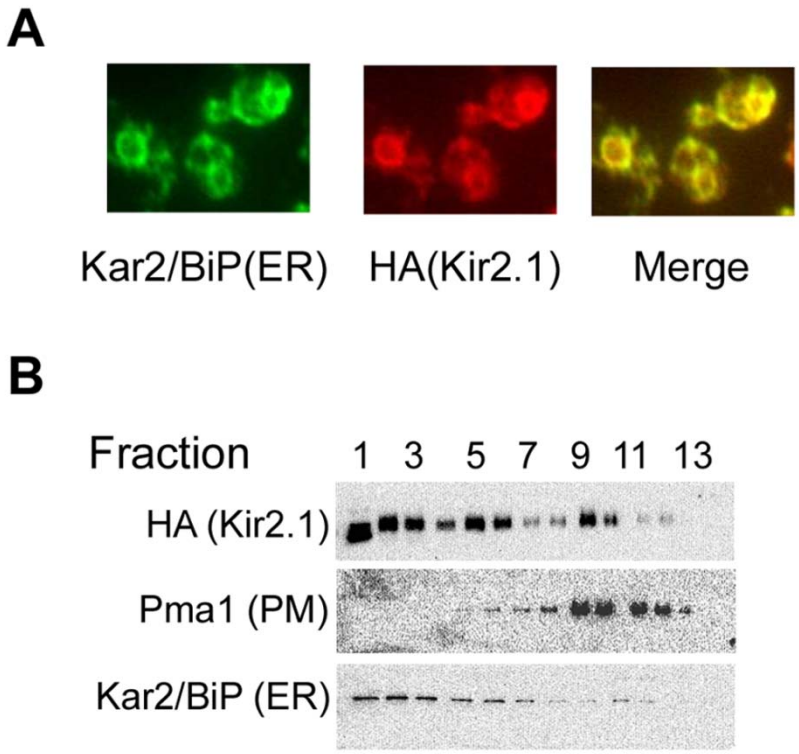


Figure 6: Kir2.1 localization in yeast.

A) Indirect immunofluorescence of yeast expressing Kir2.1. Fixed cells were probed with antibodies against the ER marker Kar2/BiP and the HA tag to visualize Kir2.1. The right panel is a merge showing extensive co-localization. B) Lysates from cells expressing Kir2.1 were analyzed by centrifugation in a 30% - 70% sucrose gradient under conditions to maximize ER and plasma membrane separation (Roberg et al., 1997). The gradient was fractionated from the top (fraction 1) to the bottom of the tubes (fraction 14). The migration of Kir2.1, the ER chaperone, Kar2/BiP, and the plasma membrane protein Pma1 were evaluated by western blot analysis.

Next, Kir2.1 protein stability in yeast was analyzed by cycloheximide chase by Patrick Needham, a post-doctoral research fellow in the Brodsky laboratory. The channel was rapidly turned-over with nearly complete degradation occurring after 90 min (Figure 7A). Kir2.1 was expressed in *pdr5Δ* yeast, which allows treatment with the proteasome inhibitor, MG132, and when this compound was added 20 min prior to the chase, Kir2.1 was partially stabilized (Figure 7A). Incomplete stabilization of Kir2.1 may be due to incomplete proteasome inhibition and/or may suggest that an alternative degradation pathway is being utilized. Further, I found that the AAA-ATPase, Cdc48, which extracts ERAD substrates from the ER membrane and into the cytosol (Wolf and Stolz, 2012), was required for degradation since Kir2.1 was completely stabilized in a temperature sensitive mutant, *cdc48-2*, at a non-permissive temperature (Figure 7B).

To determine whether Hsp70 chaperones facilitate the ERAD of Kir2.1, the protein was expressed in the temperature sensitive Ssa1 mutant, *ssa1-45*. The cytosolic Hsp70, Ssa1, is required for the degradation of ERAD substrates with misfolded cytosolic domains (Han et al., 2007; Hill and Cooper, 2000; Nakatsukasa et al., 2008; Zhang et al., 2001). Dr. Needham discovered that Kir2.1 degradation was attenuated at the non-permissive temperature in this mutant (Figure 7C). In this case, incomplete stabilization of Kir2.1 may be due to incomplete inactivation of Ssa1 or may be due to other chaperones facilitating its degradation. In contrast, Kir2.1 degradation was unaffected in a *kar2-1* mutant, which is required for the ERAD of substrates with misfolded lumenal domains (Brodsky et al., 1999; Hill and Cooper, 2000; Plemper et al., 1997) (Figure 7D). Together, these data suggest that Kir2.1 folds poorly in yeast and is recognized as an ERAD substrate by the cytosolic Hsp70, Ssa1. As described below, Kir2.1 is also targeted for ERAD in mammalian cells.

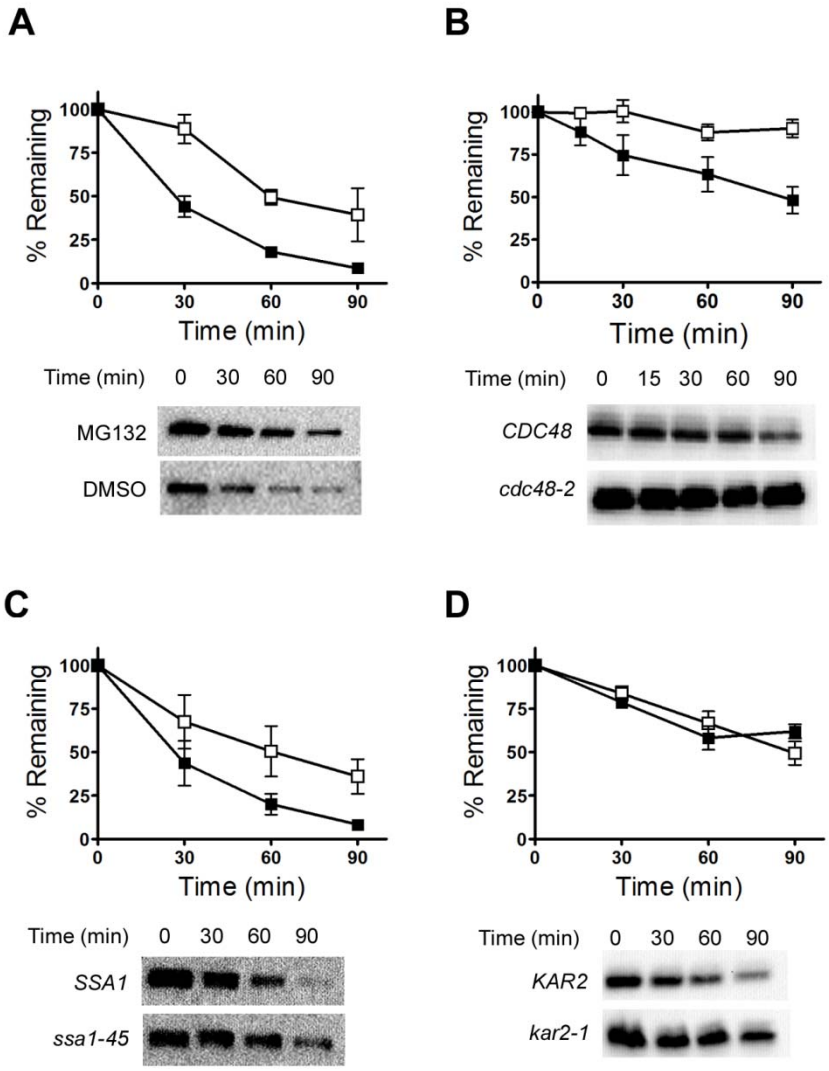


Figure 7: Kir2.1 degradation is proteasome- and cytosolic chaperone-dependent.

Cycloheximide chase reactions were performed and lysates were blotted with anti-HA antibody to measure Kir2.1 stability over time. (A) A *pdr5*Δ yeast strain was pretreated for 20 min with 100 μM MG-132 (□) or DMSO (■). Data represent the means +/- S.E. of 3 independent experiments. The degradation of Kir2.1 was also measured in (B) *CDC48* (■) or *cdc48-2* (□) mutant yeast (C) *SSA1* (■) or *ssa1–45* (□) mutant yeast, and (D) *KAR2* (■) or *kar2–1* (□) mutant yeast. Data represent the means +/- S.E. for 4–6 independent experiments. In each experiment, representative western blots are shown in the bottom panels.

Nearly all ERAD substrates in yeast are ubiquitinated by the E3 ligases, Hrd1 or Doa10 (Claessen et al., 2012). To investigate whether Kir2.1 was degraded in a Hrd1- and/or Doa10-dependent manner, Dr. Needham expressed Kir2.1 in *hrd1Δ*, *doa10Δ*, *hrd1Δ doa10Δ*, and *HRD1 DOA10* strains. He found that Kir2.1 was stabilized to a similar level when either *HRD1* or *DOA10* were deleted. When the genes encoding both E3s were absent the protein was further stabilized (Figure 8A). To verify that Doa10 and Hrd1 are directly required for Kir2.1 ubiquitination, he asked if deletion of these E3 ubiquitin ligases led to a decrease in Kir2.1 ubiquitination. Kir2.1 was immunoprecipitated from wild-type or *hrd1Δ doa10Δ* yeast and ubiquitin levels were examined by immunoblotting. Deletion of *HRD1* and *DOA10* led to a 40% reduction in Kir2.1 ubiquitination (Figure 8B-C). Because Kir2.1 degradation is Ssa1 dependent (see above), we anticipated the requirement for Doa10. However, the Hrd1 dependence suggests that Kir2.1 lesions may also be recognized within the membrane (Sato et al., 2009). Thus, the folding lesions in Kir2.1 are complex and require a set of factors that are needed for both ERAD-C and ERAD-M substrates (Carvalho et al., 2006; Denic et al., 2006; Vashist and Ng, 2004).

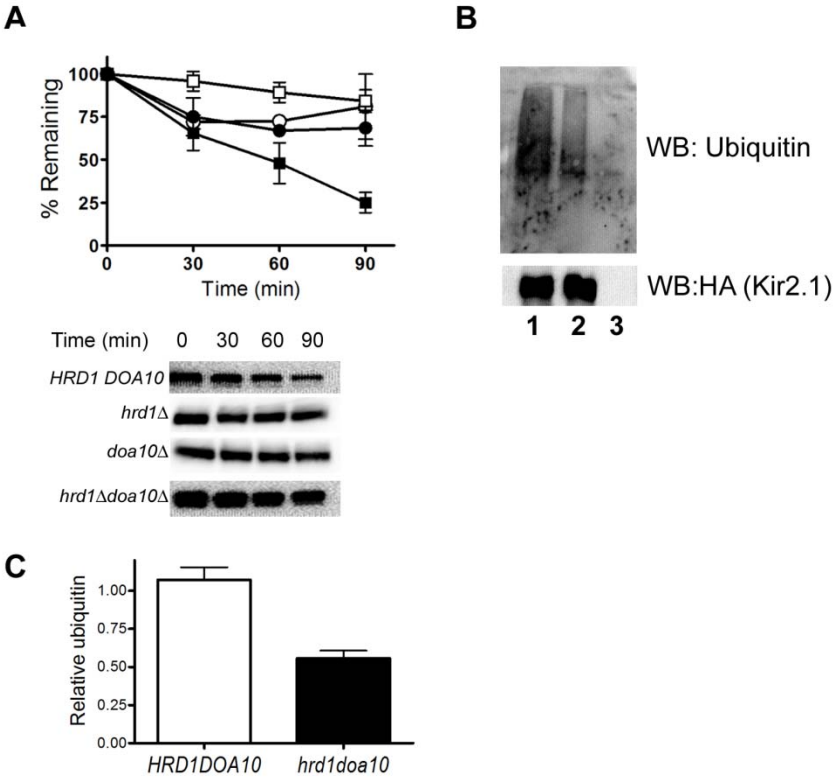


Figure 8: Kir2.1 degradation and ubiquitination are E3 ubiquitin ligase dependent.

A) Cycloheximide chase reactions were performed and lysates were blotted with anti-HA antibody to measure Kir2.1 stability over time; wild type (■), *hrd1*Δ (●), *doa10*Δ (○), or *hrd1*Δ *doa10*Δ (□). Data represent the means +/- S.E. for 4–6 independent experiments. A representative western blot from one experiment is shown below the graph. B) Kir2.1 expressed in either wild type (lane 1) or *hrd1*Δ *doa10*Δ (lane 2) mutant yeast strains was immunoprecipitated from lysates with anti-HA-agarose beads and the amount of protein and conjugated ubiquitin were analyzed by western blot (WB) analysis. The result from an immunoprecipitation using cells containing a vector control is shown for comparison (lane 3). C) Relative ubiquitination of Kir2.1 immunoprecipitated from wild type and *hrd1*Δ *doa10*Δ yeast. The graph represents the means +/- S.E. from thirteen precipitated samples of each strain, each from a distinct clone.

2.3.2 A yeast genomic screen identifies regulators of Kir2.1 biogenesis

Kir2.1 has been shown to rescue the growth of yeast strain lacking the Trk1 and Trk2 potassium transporters on medium containing low potassium (Nakamura and Gaber, 1998; Tang et al., 1995b). Because Kir2.1 is significantly subjected to ER quality control but can also transit to the plasma membrane in yeast (see above), we decided to use a genetic approach to identify the spectrum of factors that affect Kir2.1 stability in the ER and trafficking from this organelle. Moreover, because Kir2.1 is subject to quality control beyond the ER (Ma et al., 2011), I reasoned that this screen might also uncover components required for this later event. Therefore, an SGA query strain was engineered in which *TRK1* and *TRK2* were deleted. Kir2.1, under the control of various promoters, was then introduced into this strain. In principle, growth on low potassium should correlate with Kir2.1 plasma membrane residence. I ultimately determined that expressing Kir2.1 from a *TEF* promoter on a centromeric plasmid (Figure 9A) and then screening on medium lacking potassium at pH 4 (Nakamura and Gaber, 1998) and 30°C (Figure 9B) provided the optimal set of conditions to screen for genes that, when mutated, would augment growth on low potassium.

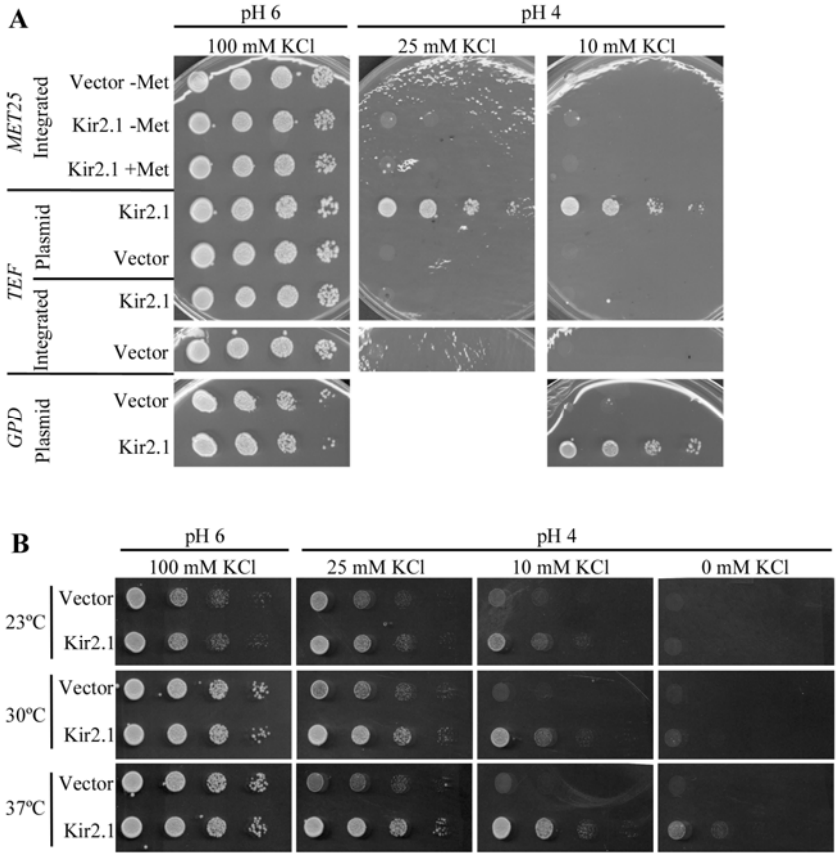


Figure 9: Optimization of Kir2.1 screening conditions

A) The *trk1Δ trk2Δ* strain expressing Kir2.1 from a *GPD*, *TEF* or *MET25* promoter were serially plated onto selective medium with 10 or 25 mM KCl at pH 4. Medium containing 100 mM KCl at pH 6 served as a control for normal growth of the strains. Where indicated, Kir2.1 was either expressed from a plasmid or integrated into the *TRK1* locus. Plates were incubated at 30°C for 5 d. B) The query strain containing a Kir2.1 expression construct under the control of the *TEF* promoter or control vector were serially plated onto selective medium with 0, 10, 25 mM KCl at pH 4 or 100 mM KCl at pH 6 at 23°C, 30°C, or 37°C and incubated for 2 d.

Preventing the ERAD of several proteins increases their folding and secretion efficiencies (Fisher et al., 2011; Grove et al., 2009; Halaban et al., 1997; Kincaid and Cooper, 2007; Mitchell et al., 1998; Qu et al., 1996; Schubert et al., 1998; Vij et al., 2006). Consistent with Kir2.1 possibly behaving in a similar manner, I found that deletion of *DOA10* subtly increased growth of the *trk1Δ trk2Δ* strain on low potassium when Kir2.1 was expressed (Figure 10). Therefore, the query strain was crossed to the yeast deletion collection using established SGA methodology (Tong and Boone, 2006). The resulting haploid strains, which each lacked one of 4,848 non-essential yeast genes were screened on selective medium at pH 4 in the absence of added potassium ($[K^+]$ < 10mM (Nakamura and Gaber, 1998)). A total of 288 strains that led to increased growth on low potassium compared to the average colony size on the plate were identified (see Figure 11A for an example). To focus my studies, strains with mutated genes that compromised select biosynthetic pathways or contained well characterized transcription and translation factors were eliminated. In addition, strains containing dubious open reading frames were eliminated from further consideration.

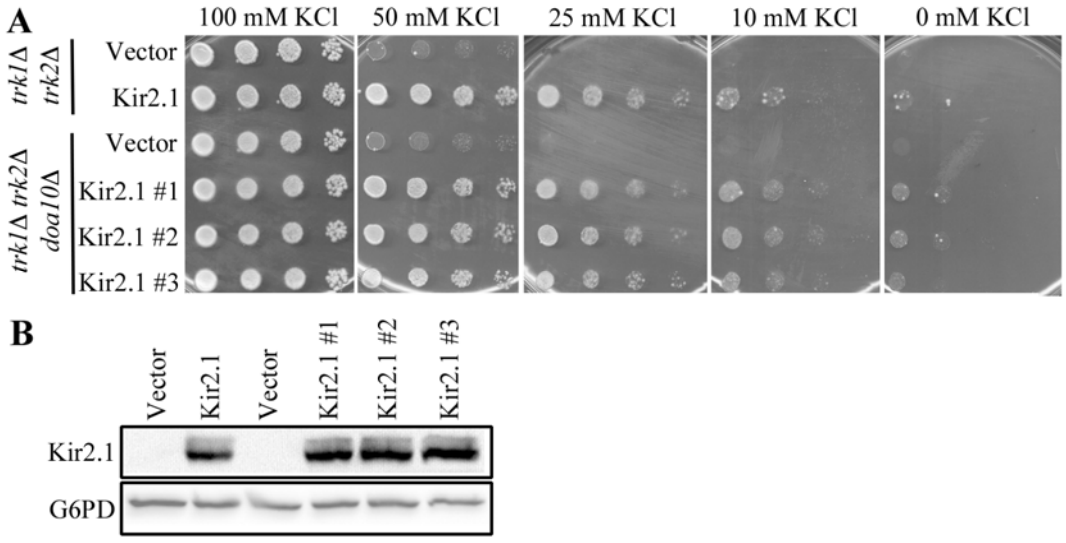


Figure 10: *doa10Δ* modestly increases Kir2.1 dependent rescue of *trk1Δ trk2Δ* on low K^+

(A) The Kir2.1 expression construct and a vector control were introduced into *trk1Δ trk2Δ* and *trk1Δ trk2Δ doa10Δ* strains, and 10-fold serial dilutions were plated onto medium with 0, 10, 25, 50, or 100 mM KCl. Three independent colonies of the *trk1Δ trk2Δ doa10Δ* strain expressing Kir2.1 are shown (#1--#3). (B) Cells were harvested from the plate, lysed, and analyzed by SDS-PAGE and western blots were probed with an anti-HA antibody to analyze Kir2.1. G6PD served as a loading control.

Fresh isolates of the remaining 215 strains that lacked unique genes were rearrayed into three 96 well plates and were again crossed to the query strain containing either a vector control or the Kir2.1 expression vector. Included in this analysis were strains lacking genes that encode for members of protein complexes that appeared to feature prominently in the preliminary screen. The strongest hits from this second screen were selected for further study, and to rank the hits, the doubling times of these strains containing either the Kir2.1 expression vector or the vector control were measured in liquid culture. The ratio of the doubling time of each strain containing the vector control to the same strain expressing Kir2.1 was then used to produce a growth score. This analysis also eliminated mutated genes that rescue growth on low potassium that were Kir2.1 independent. This left 53 deletion strains with a significantly higher growth score than the query strain (Figure 11B, Table 4, Appendix A). To my surprise, genes required for ERAD were completely absent from this list. Instead, nearly all of the genes encoded proteins associated with secretory pathway compartments within or beyond the Golgi. In fact, the corresponding proteins are primarily involved in the delivery of proteins to the vacuole, with a member of the phosphatidylinositol 3-kinase complex (Vps38) that regulates CPY sorting and interacts with retromer representing the strongest hit (Burda et al., 2002; Kihara et al., 2001). Also prominent amongst the hits were members of the ESCRT complex (Did2, Vps36, Vps22, Vps2, Vps23, Vps37, Mvb12, and Vta1).

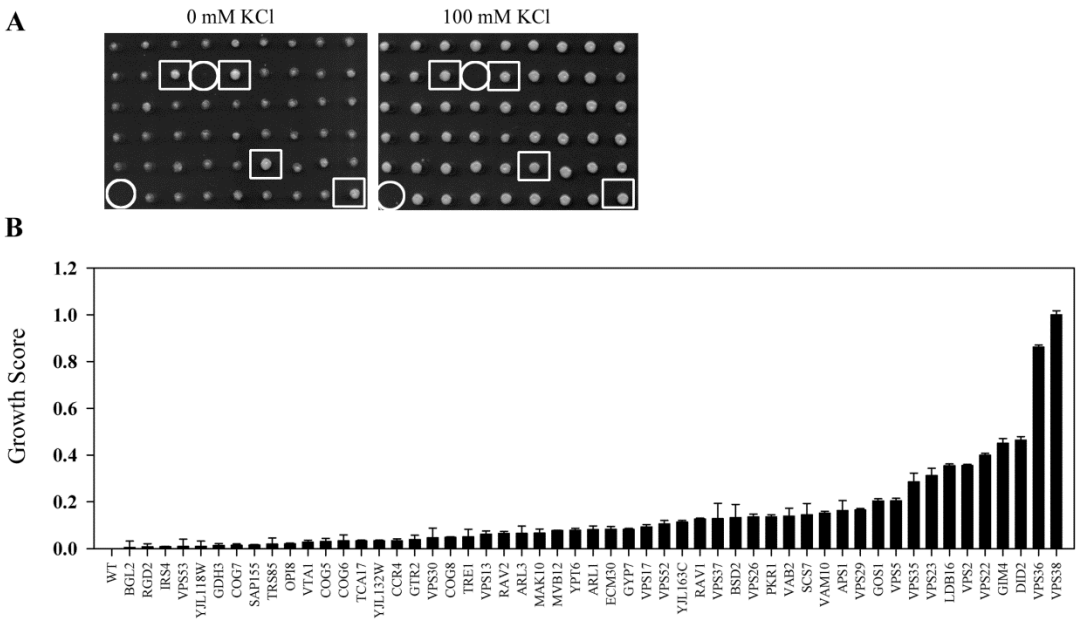


Figure 11: A genetic screen identifies ESCRT as a major effector of Kir2.1

A) Representative section of a plate following the mating of a query strain expressing Kir2.1 to the yeast deletion collection. Haploid progeny were plated on selective medium with either 0 mM KCl or 100 mM KCl, as indicated. Medium with100 mM KCl added serves as a control for equal growth amongst the different mutant strains. Circles represent negative control strains in which a strain was absent and used as a plate identifier. Boxes represent strains that showed increased growth compared to average colony growth. B) The Kir2.1 growth scores of each hit was calculated based on the doubling times of the strains with an empty vector control divided by the doubling times of strains expressing the Kir2.1 expression vector. These values were then normalized to *vps38Δ*, which was the strongest hit. All strains are deleted for *TRK1* and *TRK2,* as well as the gene indicated. Error bars represent the SD of three independent measurements.

Table 4: Genes that when deleted led to increased growth on low potassium in the *trk1Δ trk2Δ* strain

Family	Function	Genes				
Protein trafficking complexes						
ESCRT & ESCRT partners	Formation of MVBs	Vps23 Vps2	Vps37 Did2	Mvb12 Vta1	Vps22 Opi8	Vps36
PI3K II	PI3 kinase involved in vacuolar protein sorting	Vps38	Vps30			
Retromer	Retrieval of vacuolar targeted proteins	Vps5	Vps17	Vps26	Vps29	Vps35
GARP	Retrieval of vacuolar targeted proteins	Vps52	Vps54			
TRAPP	Golgi trafficking	Tca17	Trs85			
COG	Golgi cytosolic tethering complex	Cog5	Cog6	Cog7	Cog8	
RAVE	Endosomal trafficking, Golgi localization, V-ATPase assembly	Rav1	Rav2			
AP-1	Clathrin adaptor protein complex associated with protein sorting at the Golgi	Aps1				
BLOC	Endosomal sorting	Vab2				
EGO & GSE	Vacuolar invagination	Gtr2				
GTPase and associated proteins						
GTPase	Regulates trafficking through GTP hydrolysis	Arl1	Ypt6	Arl3		
GAP	Stimulates GTPase activity	Gyp7	Rgd2			
Rsp5 adaptors	Facilitate ubiquitination by the Rsp5 ubiquitin ligase	Bsd2	Tre1			
Vacuole function						
Morphogenesis	Vacuolar fusion	Vam10				
Assembly Factors	V-ATPase assembly	Pkr1				
Phosphatase activators	Stimulate dephosphorylation	Sap155	Irs4			
Other						
v-SNARE	Mediates vesicle fusion	Gos1				
Prefoldin Complex	Transfers proteins to chaperonin	Gim4				
Glutamate dehydrogenase	Synthesizes glutamate	Gdh3				
Sphingolipid alpha-hydroxylase	Long chain fatty acid biogenesis	Scs7				
NatC	Protein acetylation	Mak10				
CCR4-NOT	Transcription regulation	Ccr4				
Endo-beta-1,3-glucanase	Cell wall maintenance	Bgl2				
Uncharacterized		Ldb16 YJL118w	Vps13	Ecm30 YJL163c	YJL132w	

ESCRT is involved in Golgi and the plasma membrane quality control (Apaja et al., 2010; Wang and Ng, 2010), so I directly examined the role of select ESCRT members in Kir2.1 protein quality control. First, a *vps23Δ* strain that also lacked *TRK1* and *TRK2* was remade by PCR mutagenesis and after introduction of the Kir2.1 expression vector the yeast were rescreened on low potassium medium. Increased rescue of this strain by Kir2.1 was quite prominent compared to the trk*1Δ trk2Δ VPS23* background (Fig 11A). To confirm that rescue on low potassium medium was directly associated with Kir2.1 function, a new Kir2.1 expression plasmid was constructed. In this case, Kir2.1 contained a dominant negative mutation in which the selectivity filter was mutated from ^{144}GYG^{146} to ^{144}AAA^{146} (Tinker et al., 1996). After the plasmid was introduced into cells, the inactive channel was unable to rescue growth even though it was expressed to similar levels as wild type Kir2.1 (Figure 12A, B). Further, to ensure that Kir2.1-mediated rescue was unaffected by the presence of the HA epitope, an untagged form of Kir2.1 was expressed in *trk1Δ trk2Δ* cells and growth was rescued on low potassium medium to a similar extent as that observed for the Myc-Kir2.1-HA construct (Figure 13).

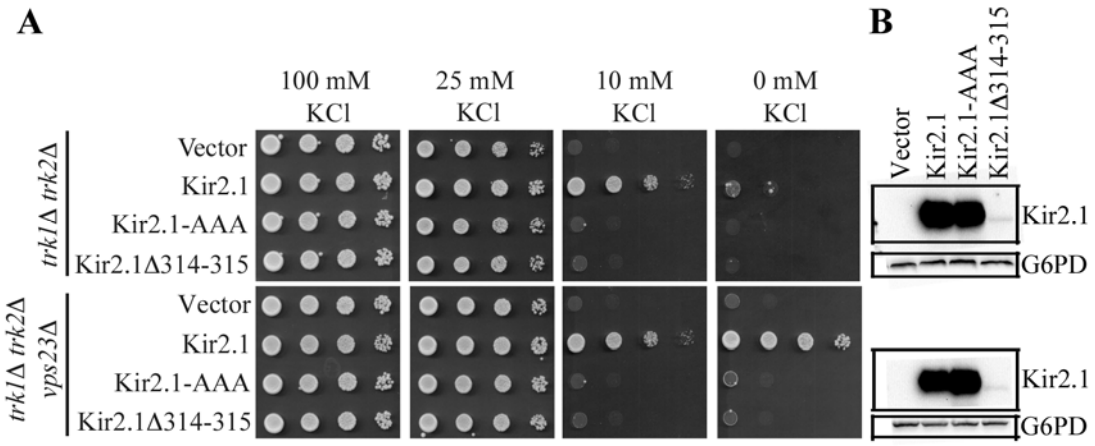


Figure 12: Deletion of *VPS23* increases Kir2.1 dependent rescue of *trk1Δ trk2Δ* on low potassium medium.

A) The control or indicated plasmids were introduced into the query strain (*trk1Δ trk2Δ*) and into a *trk1Δ trk2Δ vps23Δ* strain. 10-fold serial dilutions were plated onto selective medium with 100mM, 25mM, 10mM or 0mM KCl and growth was measured after 2 d at 30°C. B) Equal number of cells were collected from the plates, lysed, and analyzed by SDS- PAGE and blotted with anti-HA antibody to assess the levels of Kir2.1. G6PD served as a loading control.

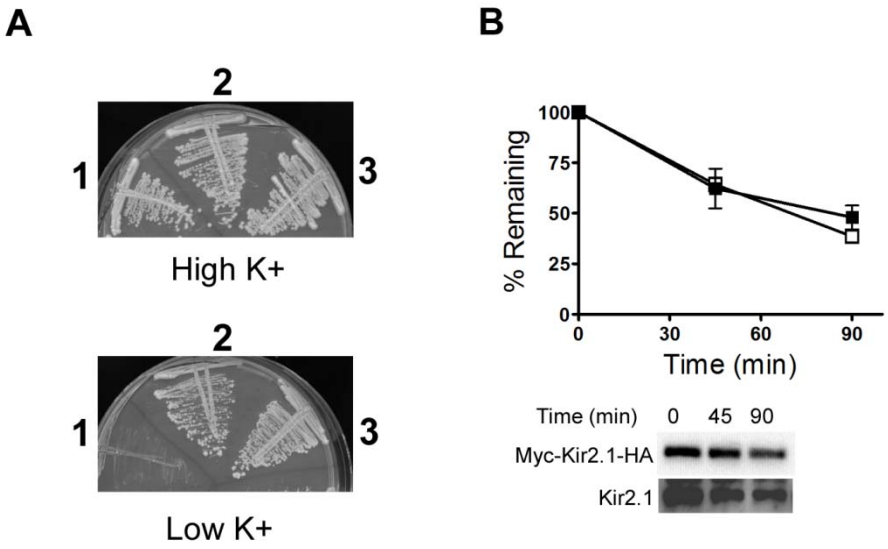


Figure 13: Affinity tagged Kir2.1 behaves similar to wild type Kir2.1.

A) Kir2.1 was expressed as either a double tagged (Myc-Kir2.1-HA) or untagged (Kir2.1) protein in *trk1trk2* yeast; empty expression vector (sector 1), doubly tagged Myc-Kir2.1-HA (sector 2), or untagged Kir2.1 (sector 3) were inoculated onto high (100mM) or low (10 mM) potassium medium and incubated for 3 d at 26°C. B) Cycloheximide chase assays were performed in wild type yeast expressing double tagged Myc-Kir2.1-HA (□) or untagged Kir2.1 (■). Data represent the means +/- S.E. for 4 independent experiments. A representative western blot from one experiment is shown below the graph.

I next wished to explore whether the yeast system could be used to assay disease linked mutations in Kir2.1 I therefore created a Kir2.1 expression vector in which a Golgi export signal was absent and that results in Andersen-Tawil syndrome in humans (Ma et al., 2011). This mutant (Δ314-315) failed to rescue growth of *trk1Δ trk2Δ* yeast on low potassium; however, the protein was expressed to lower levels than wild type Kir2.1 (Figure 13A, B). In an attempt to raise expression levels, an alternate promoter was used, but this led to increased toxicity (data not shown). To determine why Kir2.1Δ314-315 was less stable than the wild type protein, I analyzed the proteasome and vacuolar dependent degradation of Kir2.1Δ314-315 degradation. Kir2.1Δ314-315 was stabilized in the *pdr5Δ* strain when MG132 was added, but degradation was unaffected when vacuolar proteases were inactivated (Figure 14). These results suggest either that defects in the progression of the Andersen-Tawil mutant protein beyond the Golgi result in the retrieval of the protein to the ER and proteasomal degradation. Retrograde trafficking to the ER would require that the misfolded protein first bypass the ERAD machinery before it is recognized by Golgi associated factors. Alternativey, I favor a model in which the protein exhibits an ER folding defect as well as a defect in AP1-mediated trafficking (see below) resulting in recognition by ERAD components.

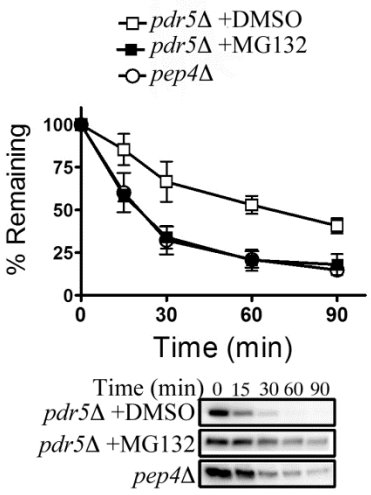


Figure 14: Kir2.1Δ314-315 is degraded by the 26S proteasome

Cycloheximide chases were performed in the *pep4Δ* strain (○) or in the *pdr5Δ* strain that was pretreated with 100 μM MG132 (□) or DMSO (■) for 30 min. Data represent the means of 5-6 independent experiments ±SD. Representative western blots from one experiment are shown.

2.3.3 Proteasome and ESCRT-dependent degradation of Kir2.1 in human cells

The results presented in the preceding sections indicate that Kir2.1 is an ERAD substrate in yeast and that later steps in the secretory pathway control the levels of active Kir2.1 at the plasma membrane. To examine whether these phenomena were evident in higher cell types, I examined the quality control of Kir2.1 in HeLa cells. Cycloheximide chase analysis may suggest that Kir2.1 was degraded by the 26S proteasome in HeLa cells, as it was in yeast, although to a much less extent (Figure 15A, B). Similar results were observed regardless of whether the cells were pretreated with MG132 (data not shown).

Since the majority of wild type Kir2.1 appeared to be degraded in a proteasome independent mechanism, the Welling lab examined whether Kir2.1 was degraded in the lysosome. Consistent with a role for lysosomal degradation in controlling Kir2.1 levels, leupeptin increased Kir2.1 steady state levels as reported (Jansen et al., 2008; Nalos et al., 2011) (Figure 15C, D). Further, leupeptin addition led to increased colocalization with the late endosomal/lysosomal marker CD63 (Figure 15E).

To determine if ESCRT was required for the lysosome dependent degradation of Kir2.1, the ESCRT components Tsg101 (ESCRT-I) and Hrs (ESCRT-0) were depleted. Treatments with targeted siRNAs led to the nearly complete absence of Tsg101 and an ~50% decrease in the amount of Hrs (Figure 15F, G). In parallel, there was a corresponding increase in Kir2.1 (Figure 15H). Addition of leupeptin did not lead to a further increase in Kir2.1 when the ESCRT components were knocked down, consistent with the delivery to and degradation of the channel in the lysosome (Figure 15I). Together, the collective data from the yeast and human cell models

demonstrate that ESCRT-dependent targeting of Kir2.1 to the vacuole/lysosome regulates the levels of functional Kir2.1.

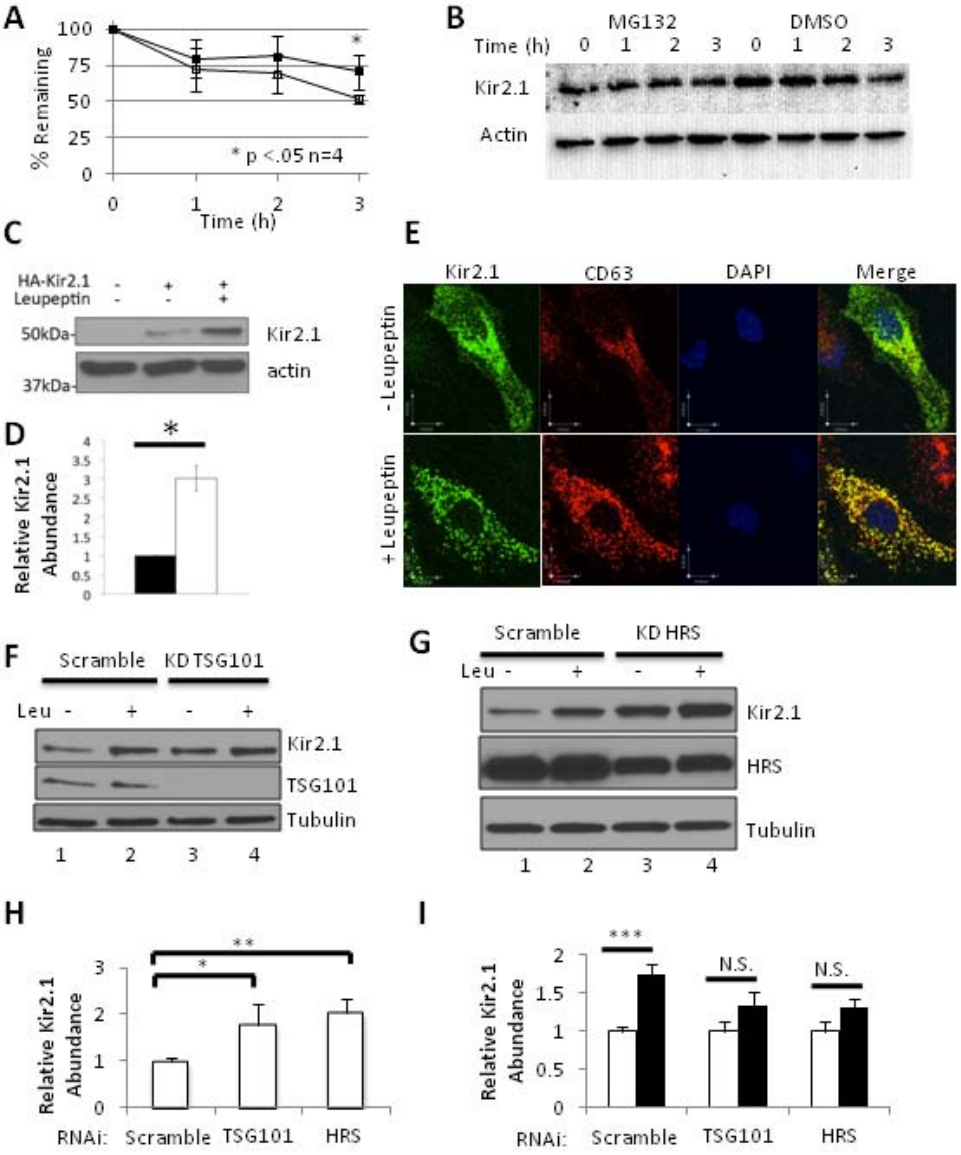


Figure 15: Kir2.1 is degradation is primarily lysosomal dependent and requires ESCRT

A) Kir2.1 stability was analyzed by a cycloheximide chase in HeLa cells. HeLa cells transfected with pcDNA3.1-HA-Kir2.1 were treated with 15μM MG132 (□) or DMSO (■) for the indicated time points and were harvested, lysed, and analyzed by SDS-PAGE. Kir2.1 was detected with

an anti-HA antibody. Actin levels served as a loading control. Data represent the means 4-5 experiments ± SD. A representative blot is shown in B. C) The amount of Kir2.1 was assessed by western blot analysis of HeLa cells transfected with pcDNA3.1- HA-Kir2.1 for 24 h followed by an 18 h treatment of 100 μM leupeptin where indicated. D) The means ± SE from 3 trials from C treated with (□) or without leupeptin (■) were quantified. E) Confocal microscopy was performed in HeLa cells transfected with pcDNA3.1-HA- Kir2.1. Cells were treated with 100 μM leupeptin as described in materials and methods and were mounted in media containing DAPI. Colocalization of Kir2.1 (green) and CD63, a marker of endosomes/ lysosomes is shown in the Merge (yellow). HeLa cells were co-transfected with the indicated siRNA and pcDNA3.1-HA-Kir2.1 and after 72h cells were harvested and the levels of Kir2.1, VPS23 VPS27, and tubulin were measured. Where indicated, 100 μM leupeptin was added to cells for 18 h. Tubulin served as a loading control. H) The levels of Kir2.1 when VPS23 or VPS27 were knocked down (lane 3 from F and G) were normalized to the scramble control (lane 1 from F and G) to determine the effect of siRNA on Kir2.1 abundance. The data represent the means ± SE of 6-8 trials. I) The levels of Kir2.1 when leupeptin was added when VPS23, VPS27, (lane 4 from F and G) or scramble (lane 2 from F and G) (■) siRNAs were transfected and were normalized to Kir2.1 levels when leupeptin was absent (lanes 1 and 3) (□) to determine the magnitude of the leupeptin response. The data represent the means ± SE of 6-8 trials. The data represent the means ± SE of 6-8 *$p<0.05$, **$p<0.01$, ***$p<0.001$

2.4 DISCUSSION

The data presented in this thesis demonstrate that Kir2.1 is subject to protein quality control at multiple levels in both yeast and human cells. An initial characterization of Kir2.1 in yeast demonstrated that a majority of the channel is ER localized and that a majority of the protein is targeted for ERAD. In contrast, our screen revealed that components required for vacuolar targeting regulate the levels of active Kir2.1. These data suggest that a larger, permanently misfolded population of Kir2.1 is targeted by ERAD and may be unable to mature. However, Kir2.1 that migrates beyond the ER appears to be in a folding competent state. At this point, ESCRT and associated factors (see below) select Kir2.1 species that may still be unfolded, but not to the extent for selection by the ERAD pathway, or they may transiently adopt misfolded conformations. The idea of an unstable population of maturing and even functional ion transporters is not new (Apaja et al., 2010; Helliwell et al., 2001; Hill and Cooper, 2000; Lu et al., 2007), but our data represent the first hint of the relative strengths of unique quality control mechanisms that act on a single protein in the secretory pathway.

To a significant extent our studies in HeLa cells validated the use of yeast as a model to identify the relative contributions of quality control pathways that impact Kir2.1 biogenesis. I show that Kir2.1 is regulated by both ERAD and by ESCRT-mediated lysosomal degradation, as in yeast, but the relative contribution of each is inverted. In yeast, the majority of Kir2.1 was degraded by ERAD with a minor population of Kir2.1 being targeted to the vacuole. In contrast, in HeLa cells Kir2.1 is primarily degraded in the lysosome, whereas a smaller population of Kir2.1 is targeted for ERAD. One explanation for this phenomenon may be the unique lipid

compositions in the ER in yeast and human cells. Of note, lipids such as cholesterol and phosphatidylinositol 4,5-bisphosphate are known to alter Kir2.1 function and may influence its stability (Huang et al., 1998; Romanenko et al., 2004). Alternatively, yeast may lack assembly or trafficking factors required for efficient transport of functional Kir2.1. For example, the rat monocarboxylate transporter, MCT1, does not efficiently traffic to the plasma membrane in yeast unless the cell surface glycoprotein, CD147, which has been shown to bind tightly to MCT1, is also expressed (Makuc et al., 2004). While yeast may not be a perfect model for studying protein quality control of mammalin proteins, the identification of ESCRT points to the utility of using such model systems.

In addition to ESCRT, we identified other factors in our yeast screen that mediate Golgi trafficking and vacuolar function. For example, Vps38, a member of the phosphatidylinositol 3-kinase complex II (PI3KII), was the strongest hit in our screen for proteins that affect Kir2.1 function. PI3K is a member of two complexes, PI3KI and PI3KII, that synthesize phosphatidylinositol 3-phosphate for a variety of functions (Kihara et al., 2001). PI3KI localizes primarily to the vacuole and is involved in autophagy, whereas PI3KII localizes to the Golgi and endosome and regulates protein trafficking from the endosome to the Golgi (Burda et al., 2002; Obara et al., 2006). Deletion of the genes encoding Vps38 and another component of both PI3K complexes, Vps30, leads to decreased retromer function (Burda et al., 2002). Retromer functions in the recovery of proteins from the endosome to the Golgi (Seaman et al., 1998), and interestingly all five members of the retromer complex (Vps5, 17, 26, 29, 35) are represented within the 20 strongest hits in the screen. Although the mechanism is undefined, deletion of retromer also leads to increased plasma membrane residence of other select membrane proteins in yeast, flies, and worms, (Dang et al., 2011; Kim et al., 2007; Korolchuk et al., 2007), which is

consistent with our findings. More recently the receptor activator of NF-κB, which is involved in osteoclast formation, was shown to be inhibited by retromer-mediated transport from the endsome to the Golgi (Xia et al., 2013). Inhibition of retromer may cause a factor that would normally antagonize Kir2.1 plasma membrane residence, such as a Golgi retention factor, to be degraded, or retromer may function in the retrieval of a Kir2.1 population that traffics through an endosomal intermediate *en route* to the plasma membrane.

Besides PI3KII and retromer, additional proteins that regulate trafficking/localization of proteins at the Golgi and endosome were identified as weaker hits in the screen. These include several Golgi-associated GTPases (Arl1, Arl3, Ypt6) that regulate Golgi-endosomal trafficking (Behnia et al., 2004; Li and Warner, 1996; Luo and Chang, 1997). For example, Ypt6, interacts with Vps52 in the Golgi-Associated Retrograde Protein (GARP) complex, which retrieves proteins from the endosome to the Golgi, and two GARP components were among the hits (i.e., Vps52 and Vps53) (Conibear and Stevens, 2000; Siniossoglou and Pelham, 2002). Other protein complexes involved in trafficking within the Golgi such as the TRAPP complex (Tca17, Trs85) (Montpetit and Conibear, 2009; Sacher et al., 2000) and the Conserved Oligomeric Golgi (COG) complex (Cog5, Cog6, Cog7, Cog8) (Ram et al., 2002; Ungar et al., 2002) were amongst the 18 weakest hits, but whether these play a direct or indirect role in Kir2.1 trafficking is unclear. Nevertheless, the regulation of Kir2.1 trafficking at the Golgi is consistent with Golgi accumulation of the Andersen-Tawil Kir2.1 mutant, which becomes trapped in this compartment due to its inability to be selected for anterograde transport (Ma et al., 2011).

Vacuole targeting of proteins via ESCRT requires ubiquitination by Rsp5 or Tul1 (Dupre et al., 2004; Helliwell et al., 2001; Hicke and Dunn, 2003; Katzmann et al., 2004; Pizzirusso and Chang, 2004; Wang et al., 2011). Rsp5 is an essential protein so it would have been absent from

our screen, but the Rsp5 adaptor proteins Bsd2 and Tre1, which regulate the trafficking and stability of plasma membrane and Golgi proteins, were identified (Hettema et al., 2004; Portnoy et al., 2000; Stimpson et al., 2006).

Importantly, the rescue of a *trk1Δ trk2Δ* strain on low potassium when each of these genes were deleted was mediated by Kir2.1 function and is not simply a result of the deleted gene making the yeast more tolerant to potassium-poor media. For each hit, I showed that the introduction of a vector control was unable to improve growth to the same level as a strain expressing Kir2.1. However, a screen for genes that when deleted increased sensitivity to cationic drugs identified Arl1, the COG complex, the GARP complex and retromer (Barreto et al., 2011), suggesting that some of the hits from our screen may alter the localization of other potassium channels, in addition to Kir2.1. These and many of our hits also lead to increased CPY secretion (Bonangelino et al., 2002), suggesting that there is general increased trafficking in strains deleted for these genes. However the strains that increase CPY secretion do not completely overlap with the hits identified in our screen, suggesting that Kir2.1 localization is regulated differently than this soluble vacuolar resident protein (see Appendix A). In fact, there is more overlap of our screen with the genes identified in a screen for genes that rescue the localization of a mutant form of the plasma membrane ATPase, Pma1, that is targeted directly from the Golgi to the vacuole (e.g. Vps38, Vps36, Vps29, Vps13, Vps35, Vps27, and Bsd2) (Luo and Chang, 1997). This result suggests the existence of an overlapping protein quality control mechanism for these two unstable plasma membrane proteins.

My data contribute to a growing number of substrates that are regulated by protein quality control at the Golgi and plasma membrane in yeast (Chang and Fink, 1995; Coughlan et al., 2004; Haynes et al., 2002; Hong et al., 1996; Jenness et al., 1997; Spear and Ng, 2003; Wang

and Ng, 2010) and mammals (Apaja et al., 2010; Armstrong et al., 1990; Ashok and Hegde, 2009; Okiyoneda et al., 2010). However, how substrates like Kir2.1 are recognized as misfolded in later compartments is not readily apparent, possibly because our screen only monitors the functional population. In contrast, Dr. Needham found that a core collection of Hsp70 and Hsp40s aid in the recognition of Kir2.1 as an ERAD substrate in the ER. Interestingly, studies in mammalian cells have identified Hsc70, Hsp90, and Hsp40s as important for the recognition of mutant CFTR that is rescued to the plasma membrane (Okiyoneda et al., 2010). Future work will uncover whether these and other chaperones operate at multiple quality control networks for Kir2.1 and perhaps other ion transporters.

3.0 DISCUSSION

Kir2.1 is a transmembrane protein that traffics through the secretory pathway, and each cellular compartment that it encounters on its way to the plasma membrane where it functions may monitor Kir2.1. A collaboration with Dr. Needham revealed that a significant portion of Kir2.1 is regulated by ERAD in yeast. However, deletion of these genes had little effect on functional levels at the plasma membrane, suggesting an alternative quality control step beyond the ER (Figure 10). In support of an additional protein quality control mechanism, the screen I performed to identify regulators of Kir2.1 plasma membrane residence primarily revealed endosomal/Golgi proteins (Figure 11, Table 4 Appendix A). The fact that ERAD genes were not identified suggests a division of labor for Kir2.1 regulation in which ERAD monitors the nonfunctional protein while factors later in the secretory pathway monitor the protein that may still function.

Kir2.1 is an ERAD substrate in both yeast and mammals (Figures 7 and 15), but it requires a set of factors that are not solely represented by ERAD-L, ERAD-C, or ERAD-M (see Section 1.4). Specifically, its degradation is dependent on Hrd1, but not Kar2/BiP, which represents the ERAD-L/M pathways. In addition, Doa10 and Ssa1 of the ERAD-C pathway are required for the degradation of Kir2.1. The degradation dependence on multiple ERAD pathways seems to be a developing theme among multimeric ion channels. For example, ENaC, another multimeric channel with similar topology to Kir2.1, also shows dependence on both the Hrd1

and Doa10 E3 ubiquitin ligases (see Appendix B). Both of these ion channels are transmembrane proteins with a cytosolic loop, and could have misfolded lesions that can be recognized by both Hrd1 and Doa10. This hints at multiple lesions within Kir2.1 that are recognized by the ERAD machinery.

These lesions may result in part from the channel monomer's inefficient assembly into a tetrameric channel. Kir2.1 requires contacts within the membrane and the ER lumen that appear to be important for its oligomerization (Tinker et al., 1996) and the exposure of these contacts in the absence of assembly may reveal ERAD recognition sites. Alternatively, assembly may be required to expose its ER export signal and/or mask an unidentified ER retention signal, similarly to what has been observed with other Kir channel localization signals (Ma et al., 2011; Yuan et al., 2003). A poorer assembly efficiency in yeast may also explain why there is more Kir2.1 ERAD than in mammalian cells.

Even though Kir2.1 is primarily an ERAD substrate in yeast, the screen I performed identified primarily Golgi/endosomal-associated proteins (Appendix A), suggesting a post ER regulation of Kir2.1, which is consistent with the Andersen-Tawil mutant accumulating in the Golgi in mammalian cells. In addition, the identification of ESCRT indicates that Kir2.1 is targeted to the vacuole. However, it is also possible that ESCRT is indirectly affecting Kir2.1 stability by targeting another factor that alters channel activity. Studies in human cells confirm that Kir2.1 is stabilized when the lysosome is degraded suggesting lysomal degradation of Kir2.1 (Jansen et al., 2008). However, it is not clear whether the cellular decision to degrade Kir2.1 is being made at the plasma membrane or in an intracellular compartment.

The large number of Golgi/endosomal proteins identified would suggest that the decision to degrade Kir2.1 is made at an intracellular compartment. The identification of retrieval

complexes such as retromer and GARP (Table 4, Appendix A) is consistent with a decision being made at the endosome. However, studies in collaboration with the Welling lab demonstrated that a reduction of retromer did not alter total Kir2.1 protein levels (data not shown), suggesting that if retromer also functions to regulate Kir2.1 in human cells it does so by redistributing Kir2.1 from a plasma membrane directed pool to an intracellular resident pool. One possibility is that GARP and retromer retrieve a population of Kir2.1 in endosomes that would otherwise traffic to the plasma membrane. If this is the case, then an examination of Kir2.1 plasma membrane levels by biotinylation in HeLa cells could reveal that there is more Kir2.1 at the plasma membrane when retromer is inhibited. Moreover, if deletion of retromer components simply alters the distribution of Kir2.1 and disruption of ESCRT prevents Kir2.1 degradation, then deletion of both ESCRT and retromer components in the context of *trk1Δ trk2Δ* should produce synergistic growth on low potassium in yeast.

Although I have identified factors that regulate Kir2.1 and there are hints as to where they act, it is not clear if the decision to degrade Kir2.1 is made before or after delivery to the plasma membrane. Retromer may be targeting a pool of Kir2.1 being delivered to the plasma membrane, or it may be targeting a recycling population following internalization. To determine if factors such as retromer recognize Kir2.1 before or after delivery to plasma membrane, a mutant Kir2.1 construct in which a tyrosine based motif important for endocytosis is impaired could be used (Tong et al., 2001; Wischmeyer et al., 1998). This mutant should increase plasma membrane residence of Kir2.1 and should increase growth on low potassium in a *trk1Δ trk2Δ* strain due to Kir2.1 being trapped at the plasma membrane. If this mutant is combined with the deletion of a gene product that acts prior to endocytosis (e.g., retromer), then there should be increased growth, whereas if it acts after endocytosis there should be no further increased

growth. Alternatively, deletion of *END3* inhibits endocytosis (Benedetti et al., 1994) and if combined with deletion of a gene that alters Kir2.1 plasma membrane residence there should be a similar result as found in experiments with the tyrosine mutant. While using an *end3* mutant strain would provide less specificity to Kir2.1, it would also inhibit the growth rate of the yeast strain, thus, allowing for an increased signal of Kir2.1 mediated rescue of *trk1Δ trk2Δ* upon deletion of a regulatory factor that functions prior to endocytosis.

Although the functional rescue of the *trk1Δ trk2Δ* strain by Kir2.1 indicates that it is functioning at the plasma membrane, the only endocytic factors that were identified were the Rsp5 adaptors, Tre1 and Bsd2, suggesting that Kir2.1 is ubiquitinated by Rsp5. An Rsp5 temperature sensitive mutant that is catalytically inactive provides a tool to determine whether either of these adaptors do in fact lead to the Rsp5 dependent ubiquitination of Kir2.1 (Wang et al., 1999). However, Rsp5 also functions at the Golgi and ER (Haynes et al., 2002; Shcherbik et al., 2003; Wang et al., 2011), and the large number of hits associated with the Golgi may suggest that these genes act there. While studies of how Tre1 and Bsd2 regulate Kir2.1 may reveal a mechanism for targeting Kir2.1 for degradation in yeast, translating these findings to human cellular biology is currently infeasible because both of these genes lack clear mammalian homologs.

Kir2.1 is degraded in the yeast vacuole, but genes that control vacuolar function such as the vacuolar protease, Pep4, were not identified. One possibility is that the vacuole represents a terminal site of protein trafficking and that once delivered to the vacuole a protein becomes trapped; thus, Kir2.1 would not be able to traffic back to the plasma membrane where it functions. In this model, Kir2.1 would accumulate in the vacuole when genes important for vacuolar function are deleted. However, this is difficult to test in yeast because the majority of

Kir2.1 is ER localized. Nevertheless, I did attempt to see if Kir2.1 accumulates in the vacuole in a *pep4Δ* strain by immunofluorescence. Unfortunately due to low signal, I saw inconclusive weak accumulation of Kir2.1 in the vacuole (data not shown).

In fact it takes very few active channels to rescue growth of the potassium transporter deficient strain. While Trk1 is estimated to be ~8 fold more abundant than Trk1, both proteins are ranked in the bottom 5% of expressed proteins in *S. cerevisiae* (Wang et al., 2012). Therefore, it is not suurprinsing that it does not take much Kir2.1 to rescue growth on low potassium. For example, if it is assumed that a yeast cell has a volume of 100 fL, a membrane potential of -75 mV and requires 100 mM internal concentration of potassium to survive, that Kir2.1 has a single channel conductance of 30 ps and an open probability of 0.7, and that the membrane potential is not altered by the expression of Kir2.1, then it would take ~10 min for one channel to restore the intracellular potassium concentration from 1 mM to 100 mM on 10 mM potassium (Welling, PA., 2013 personal correspondence). If so few channels are required to see a phenotype, then localization studies to determine organellar distribution in yeast are infeasible.

The small amount of Kir2.1 needed to produce the growth phenotype in *trk1Δ trk2Δ* yeast limits the ability to perform biochemical studies. For example, indirect immunofluorescence of Kir2.1 an overwhelming ER signal masks the signal at the plasma membrane even when ESCRT is deleted. Even when Kir2.1 was expressed from the highly expressing GPD promoter, sucrose gradient centrifugation suggested that less than 5% of Kir2.1 resided at the plasma membrane (Figure 6), and monitoring changes in such a small population is difficult and makes such studies in yeast infeasible. Therefore, the need arises for alternate systems to elucidate the factors that regulate Kir2.1 biogenesis.

Evaluating the hits from the screen in mammalian expression systems such as HeLa cells provides a more physiologically relevant system to study human potassium channels and was used to confirm ESCRT's role in regulating Kir2.1 plasma membrane residence. However, to evaluate proteins identified in yeast the identity of the human homolog must be known. As discussed yeast offer numerous advantages (see Section 1.1), and a yeast expression system that increases Kir2.1 plasma membrane residence would be a valuable tool to study post-ER quality control of Kir2.1. In one established system, Kir2.1 was expressed from the inducible, high expressing *GAL* promoter. The increased plasma membrane residence observed in this system may be a result of the inducible system. Kir2.1 may take time to accumulate to the point that it is recognized by ERAD and acute expression of the protein may prevent such accumulation. Alternatively, high expression of Kir.21 may saturate ERAD, similar to what is observed when the ERAD substrate, CPY*, is overexpressed, and is instead targeted to the vacuole (Haynes et al., 2002). This presents an added challenge because if protein quality control becomes saturated, then it becomes unclear if the amount of degraded protein increases from the "normal" protein quality control pathways or if cellular morphology has been altered. If native levels of protein quality control are still occurring, inducible yeast systems like the one that utilizes the *GAL* promoter may be better suited for characterizing gene that act after ERAD. While this system may lead to more plasma membrane residence and provide a way to biochemically characterize hits from the screen, inducible expression systems were unsuccessful in performing screens of Kir3.2 due to the low signal to noise (Haass, 2007).

Several uncharacterized and poorly characterized genes were identified in the yeast screen I performed that would be more easily studied in alternative expression systems that increase Kir2.1 plasma membrane residence. One protein of particular interest is Vps13

(Appendix A). Vps13 has been identified in other screens to identify factors that regulate the trafficking of secretory proteins (Brickner and Fuller, 1997; Chang and Fink, 1995; Redding et al., 1996; Robinson et al., 1988; Rothman et al., 1989). Vps13 is a Golgi localized protein, whose human ortholog has been linked to acanthocytosis and Cohen syndrome disease (Kolehmainen et al., 2003; Rampoldi et al., 2001). In yeast, deletion leads to the accumulation of intralumenal vesicles and decreased phosphoinositides at the prospore, leading to decreased sporulation efficiency (Park and Neiman, 2012). Decreased sporulation efficiency is also shared with ESCRT members, such as Vps27 (Deutschbauer et al., 2002).

Like Vps13, the function of ECM30 (Appendix A) is unknown but data suggest it may alter Gap1 sorting and show cell wall defects, possibly due to the mislocalization of one of biosynthetic components (Costanzo et al., 2010; Lussier et al., 1997). In addition, the open reading frame YJL163C which shows homology to folate transporter, and YJL132W, which shows homology to the glycosylphosphatidylinositol phospholipase D1 gene were identified.

Besides understanding how genes are involved in trafficking, the genetic screen I performed provides a model for understanding protein quality control mechanisms of other potassium channels. There are four other members of the Kir2 subfamily and six other Kir families that could be potentially used in the screen I developed. Although these proteins are closely related, they differ in function, cellular localization, and protein interactions, suggesting that they may be regulated differently (Hibino et al., 2010). For example, Kir1.1 (ROMK) is primarily ER localized in human cells and thus may be more dependent on ERAD (Yoo et al., 2005). However, ROMK is unable to rescue growth of the potassium transporter deficient strain on low potassium unless a point mutation is made in the pH sensing domain (Paynter et al., 2010), suggesting that such screens may not be as straight forward.

In addition to Kir proteins, there are numerous potassium channels of physiological relevance that can be screened. Besides potassium channels, the selectivity filter of sodium channels can be mutated to allow them to be more potassium permissive, and I have attempted to perform the screen with the epithelial sodium channel (see Appendix B). Although I was unsuccessful, mutations in the selectivity filter of Kir3.2 to make it sodium permissive suggest that such a strategy may be successful. In conclusion, the screen I performed establishes a model to probe the protein quality control of ion channels, and may lead to a better understanding of how the cell monitors proteins as they progress through the secretory pathway. The efforts may also ultimately lead to a way to treat the many channelopathies that exist.

APPENDIX A

SCREEN HITS

Table 5: Expanded list of genes identified in a screen for increased Kir2.1 funciton

	Gene Name	SGD Description [a]	Summaries [b]	Reference	Mammalian Ortholog	Growth Score [c]	CPY Secretion [d]
1	*VPS38*	Part of a Vps34p phosphatidylinositol 3-kinase complex that functions in carboxypeptidase Y (CPY) sorting; binds Vps30p and Vps34p to promote production of phosphatidylinositol 3-phosphate (PtdIns3P) which stimulates kinase activity	Identified in VPL screen that mislocalizes vacuolar proteins to the cell surface	(Rothman et al., 1989)	UVRAG (Itakura et al., 2008)	1	0.97
			Involved in targeting carboxypeptidase Y (CPY) and mutant Pma1 to the vacuole	(Luo and Chang, 1997)			
			Mediates interaction between Vps30 and Vps34-Vps14 - PI3K complex II	(Kihara et al., 2001)			
			Involved in targeting CPY and not Pma1 or CPS, Vps5 and Vps17 (retromer) bind PI3P via PX domain	(Burda et al., 2002)			
2	*VPS36*	Component of the ESCRT-II complex; contains the GLUE (GRAM Like Ubiquitin binding in EAP45) domain which is involved in interactions with ESCRT-I and ubiquitin-dependent sorting of proteins into	Identified in VPL screen that mislocalizes vacuolar proteins to the cell surface, forms class E compartment,	(Rothman et al., 1989)	EAP45/VPS36 (Kamura et al., 2001)	0.86	0.58
			Identified in screen that led to increased alkaline phosphatase (ALP) in the vacuole, increased CPY secretion,	(Nothwehr et al., 1996)			

the endosome	Forms a complex with Vps36 and Vps22 by gel filtration, associates with endosomes by fluorescent microscopy, release from membranes is Vps4 dependent, functions downstream of ESCRTI and binds to ESCRTIII	(Babst et al., 2002b)
	Crystal structure of quaternary ESCRT II complex shows stoichiometry of 2:1:1 of Vps25:Vps22:Vps36, hydrophobic patch of Y441, L97, I100, L141 important for binding to Vps22, disruption of complex leads to class E phenotype, D548 forms a salt bridge with R83 of Vps25,	(Hierro et al., 2004)
	Crystal structure of quaternary ESCRT II complex shows stoichiometry of 2:1:1 of Vps25:Vps22:Vps36, four salt links with Vps22, ESCRTII interacts with Vps20 (ESCRTIII) which increases binding of ESCRTIII to membranes *in vitro*	(Teo et al., 2004)
	Identified as a suppressor of *cdc1-1*, suppression due to accumulation of Smf1, conferred sensitivity to Li+ and Ca2+	(Eguez et al., 2004)
	The N-terminal GLUE domain interact with the C-terminus of Vps28 by gel filtration and co-immunoprecipitation, ESCRTII complex binds to Folch lipid vesicles mediated by the GLUE domain which contains a PH domain, GLUE domain localizes ESCRTII to endosomes, Vps36 NZF domain binds ubiquitin	(Teo et al., 2006)

3	*DID2*	Class E protein of the vacuolar protein-sorting (Vps) pathway; binds Vps4p and directs it to dissociate ESCRT-III complexes; forms a functional and physical complex with Ist1p; human ortholog may be altered in breast tumors	Class A (wild-type) vacuoles in vacuole morphology study	(Raymond et al., 1992)	CHMP1 (Howard et al., 2001)-CHMP1A CHMP1B (Tsang et al., 2006)	0.46	0.07
			Identified as a suppressor of *doa4* mutant, characterized similar to Vps46, localizes to class E compartment when Vps4/Did6 ATPase is defective	(Amerik et al., 2000)			
			Did2 coordinates Vps4-mediated dissociation of ESCRT-III from endosomes	(Nickerson et al., 2006)			
			Ist1, Did2, Vta1, and Vps60 Function Together to Positively Modulate Cargo Sorting, Did2 recruits Ist1 to endosomes	(Rue et al., 2008)			
4	*GIM4*	Subunit of the heterohexameric cochaperone prefoldin complex which binds specifically to cytosolic chaperonin and transfers target proteins to it	Synthetically lethal with *TUB4*, deletion is osmotically sensitive	(Geissler et al., 1998)	PFD2 (Vainberg et al., 1998)	0.45	0.04
			Part of prefoldin complex that binds chaperonin	(Vainberg et al., 1998)			
			Synthetically lethal with *STU1*	(Brew and Huffaker, 2002)			
5	*VPS22*	Component of the ESCRT-II complex, which is involved in ubiquitin-dependent sorting of proteins into the endosome; appears to be functionally related to SNF7; involved in glucose derepression	Identified in VPT screen which led to increased secretion of CPY-invertase fusion protein,	(Robinson et al., 1988)	EAP30 (Schmidt et al., 1999)	0.40	0.54
			Identified in VPL screen that mislocalizes vacuolar proteins to the cell surface	(Rothman et al., 1989)			
			Identified in mutagenesis screen for inability to grow on raffinose	(Vallier and Carlson, 1991)			
			Forms a complex with Vps36 and Vps22 by gel filtration, associates with endosomes by fluorescent microscopy, release from membranes is vps4 dependent, functions downstream of ESCRTI and binds to ESCRTIII	(Babst et al., 2002b)			

			Crystal structure of quaternary ESCRTII complex shows stoichiometry of 2:1:1 of Vps25:Vps22:Vps36, coiled coil with 2 winged helix domains, binds to hydrophobic patch of Vps36 (Y441, L97, I100, L141)	(Hierro et al., 2004)			
			Crystal structure of quaternary ESCRTII complex shows stoichiometry of 2:1:1 of Vps25:Vps22:Vps36, four salt links with Vps36, D214 form a salt bridge with vps25 R83, ESCRTII interacts with vps20 (ESCRTIII) which increases binding of ESCRTIII to membranes *in vitro*	(Teo et al., 2004)			
			Identified as a suppressor of *cdc1-1*, suppression due to accumulation of Smf1, conferred sensitivity to Li+ and Ca2+	(Eguez et al., 2004)			
6	*VPS2*	Class E Vps protein of the ESCRT-III complex, required for sorting of integral membrane proteins into lumenal vesicles of multivesicular bodies, and for delivery of newly synthesized vacuolar enzymes to the vacuole, involved in endocytosis	Identified in a screen for increased CPY secretion	(Rothman and Stevens, 1986)	CHMP2a (Fujita et al., 2004)	0.36	0.37
			Identified in screen for defective endocytosis of STE3	(Davis et al., 1993)			
			Identified in screen that led to increased ALP in the vacuole, increased CPY secretion,	(Nothwehr et al., 1996)			
			Identified in VPT screen which led to increased secretion of CPY-inveratase fusion protein,	(Robinson et al., 1988)			
			Identified as a suppressor of *doa4* mutant, characterized similar to vps46, localizes to class E compartment when vps4/Did6 ATPase is defective	(Amerik et al., 2000)			
			Forms ESCRTIII complex with Vps20, vVs24 and snf7 by	(Babst et al., 2002a)			

			fractionation and colocalization, forms a subcomplex with vps24				
7	*LDB16*	Protein of unknown function; null mutants have decreased net negative cell surface charge; GFP-fusion protein expression is induced in response to the DNA-damaging agent MMS; native protein is detected in purified mitochondria	Screen of ldb phenotype that is associated with reduced incorporation of mannosyl phosphate groups into the mannoprotein-linked oligosaccharides	(Corbacho et al., 2005)	none	0.35	0
			Mitochondria proteomics	(Reinders et al., 2006)			
			Microarray reveals at least three fold increase in response to DNA damage (MMS)	(Lee et al., 2007)			
			Removal of promoter produces weak Opi phenotype	(Hancock et al., 2006)			
8	*VPS23*	Component of the ESCRT-I complex, which is involved in ubiquitin-dependent sorting of proteins into the endosome; homologous to the mouse and human Tsg101 tumor susceptibility gene; mutants exhibit a Class E Vps phenotype	Identified in VPL screen that mislocalizes vacuolar proteins to the cell surface, normal vacuole	(Rothman et al., 1989)	Tsg101 (Li et al., 1999)	0.31	0.67
			Deletion rescues mating defect of *stp2* mutant, localizes to class E compartment	(Li et al., 1999)			
			Cloned by complementation, interacts with vps28, CPS misprocessing suggests endosomal function	(Babst et al., 2000)			
			Characterization of ESCRT1 (Vps23,28,37), binds ubiquitinated cargo	(Katzmann et al., 2001)			
9	*VPS35*	Endosomal subunit of membrane-associated retromer complex required for retrograde transport; receptor that recognizes retrieval signals on cargo proteins, forms subcomplex with Vps26p and Vps29p that selects cargo proteins for retrieval; interacts with Ypt7p	Identified in VPT screen which led to increased secretion of CPY-invertase fusion protein	(Bankaitis et al., 1986; Robinson et al., 1988)	Vps35 (Zhang, 2000)	0.29	0.99
			Identified in screen that led to increased ALP in the vacuole, increased CPY secretion	(Nothwehr et al., 1996)			
			Dominant negative is rescued by overexpression of Vps29, fractionates with Vps29, required for assembly of the complex, forms a subcomplex with Vps26	(Seaman et al., 1998)			

			and Vps29, primarily membrane bound, deletion causes disruption of Vps29 membrane association, crosslinking experiment pulls down rest of retromer				
			Crosslinks with A-ALP, used to examine retromer sorting signal	(Nothwehr et al., 2000)			
10	*VPS5*	Nexin-1 homolog required for localizing membrane proteins from a prevacuolar/late endosomal compartment back to the late Golgi apparatus; structural component of the retromer membrane coat complex; forms a retromer subcomplex with Vps17p; required for recruiting the retromer complex to the endosome membranes	Identified in VPT screen which led to increased secretion of CPY-inveratase fusion protein,	(Bankaitis et al., 1986; Robinson et al., 1988)	SNX1 (Nothwehr, 1997), Snx2 (Schwarz, 2002) (Wassmer, 2009)	0.20	0.97
			Deletion exhibits class B vacuolar morphology	(Raymond et al., 1992)			
			Identified in screen that led to increased ALP in the vacuole, increased CPY secretion, mutant has growth defect at 37°C	(Nothwehr et al., 1996)			
			Crosslinking experiment pulls down rest of retromer, is able to self assemble,	(Seaman et al., 1998)			
11	*GOS1*	v-SNARE protein involved in Golgi transport, homolog of the mammalian protein GOS-28/GS28	Gos1 identified as a SNARE *in silico*	(McNew et al., 1997)	GOS-28/GS28 (McNew et al., 1998)	0.20	0.53
			A snare that directly associates with Sed5, deletion disrupts ER-Golgi or intra-Golgi transport of CPY	(McNew et al., 1998)			
12	*VPS29*	Endosomal protein that is a subunit of the membrane-associated retromer complex essential for endosome-to-Golgi retrograde transport; forms a subcomplex with Vps35p and Vps26p that selects cargo proteins for endosome-to-Golgi retrieval	Identified in VPT screen which led to increased secretion of CPY-inveratase fusion protein	(Bankaitis et al., 1986; Robinson et al., 1988)	Vps29 (Haft et al., 2000)	0.17	1
			Rescues a dominant negative Vps35 suggesting an interaction, fractionates with Vps35, membrane localization is dependent on Vps35, crosslinking experiment pulls down rest of retromer, required for assembly of the complex, forms a subcomplex with Vps26 and Vps35	(Seaman et al., 1998)			

13	*APS1*	Small subunit of the clathrin-associated adaptor complex AP-1, which is involved in protein sorting at the trans-Golgi network; homolog of the sigma subunit of the mammalian clathrin AP-1 complex	Identified in yeast by degenerate PCR, deletion with CHC1 caused growth defect, Aps1 associates with a biochemically distinct complex from Aps2	(Phan et al., 1994)	AP19/AP1S1, AP17/AP1S2 (Kirchhausen et al., 1991), AP1S3	0.16	0.01
14	*VAM10*	Protein involved in vacuole morphogenesis; acts at an early step of homotypic vacuole fusion that is required for vacuole tethering	Deletion prevents vacuolar fusion under osmotic conditions, displays Vam3 dependent vacuolar fusion *in vitro*, Opposite of *VPS5* strand	(Kato and Wickner, 2003)	none	0.15	0.94
15	*SCS7*	Sphingolipid alpha-hydroxylase, functions in the alpha-hydroxylation of sphingolipid-associated very long chain fatty acids, has both cytochrome b5-like and hydroxylase/desaturase domains, not essential for growth	Identified as being similar to cytochromeb5, required for α-HO 26:0	(Mitchell and Martin, 1997)	FA2H (Alderson et al., 2004)	0.15	0.02
16	*VAB2*	Protein with a potential role in vacuolar function, as suggested by its ability to bind Vac8p; likely member of BLOC complex involved in endosomal cargo sorting; Vab2p-GFP-fusion localizes to cytoplasm in punctate pattern	Interacts with Vac8 by yeast two hybrid, Vac8 and Nvj1 mediate nucleus-vacuole junctions Localizes to cytoplasm in punctate pattern Vab2 interacts with members of the BLOC-1 complex (sorts cargo to lysosome related organelles)	(Pan et al., 2000) (Huh et al., 2003) reviewed in (Hayes et al., 2011)	none	0.14	0.02
17	*PKR1*	V-ATPase assembly factor; functions with other V-ATPase assembly factors in the ER to efficiently assemble the V-ATPase membrane sector (V0); protein abundance increases in response to DNA replication stress	Deletion causes growth defect on low iron media due to Fet3 (iron transporter) activity at the plasma membrane, vacuole ATPase decreased in deletion preventing vacuole acidification, ER localized	(Davis-Kaplan et al., 2006)	none	0.14	0.47
18	*VPS26*	Vacuolar protein component of the retromer; forms part of the multimeric membrane-associated	Identified in VPT screen which led to increased secretion of CPY-inveratase fusion protein	(Bankaitis et al., 1986; Robinson et al., 1988)	VPS26A, VPS26B (Kerr et al., 2005)	0.14	1

		retromer complex involved in vacuolar protein sorting along with Vps35p, Vps29p, Vps17p, and Vps5p; essential for endosome-to-Golgi retrograde protein transport; interacts with Ypt7p; protein abundance increases in response to DNA replication stress	Identified in screen that led to increased ALP in the vacuole, increased CPY secretion,	(Nothwehr et al., 1996)			
			Crosslinking experiment pulls down rest of retromer, mislocalizes Vps10 similar to other retromer components, forms a subcomplex with Vps29 and Vps35	(Seaman et al., 1998)			
19	*BSD2*	Heavy metal ion homeostasis protein, facilitates trafficking of Smf1p and Smf2p metal transporters to the vacuole where they are degraded, controls metal ion transport, prevents metal hyperaccumulation, functions in copper detoxification	Mutant has increased sensitivity to copper and cadmium,	Liu, 1994	none	0.13	0.03
			Localizes to the ER, mutants are hypersensitive to cadmium and copper due to loss of regulation of Smf1,	(Liu et al., 1997)			
			Regulates Smf2 similar to Smf1	(Portnoy et al., 2000)			
20	*VPS37*	Component of the ESCRT-I complex, which is involved in ubiquitin-dependent sorting of proteins into the endosome; suppressor of rna1-1 mutation; may be involved in RNA export from nucleus	Identified in VPL screen that mislocalizes vacuolar proteins to the cell surface	(Rothman et al., 1989)	VPS37A, VPS37B, VPS37C, VPS37D (Seaman, 2008)	0.13	0.83
			characterization of ESCRT1 (Vps23,28,37), binds ubiquitinated cargo	(Katzmann et al., 2001)			
21	*RAV1*	Subunit of the RAVE complex (Rav1p, Rav2p, Skp1p), which promotes assembly of the V-ATPase holoenzyme; required for transport between the early and late endosome/PVC and for localization of TGN membrane proteins; potential Cdc28p substrate	Identified in screen that rescued mating competency of Kex2 mutant (Soi3), mutants displayed normal vacuolar morphology, interferes with Kex2 retention	(Redding et al., 1996)	DMXL1 (Kraemer et al., 2000)	0.13	0.24
			Associated with Skp1 and Rav2, associates with members of the Vacuolar ATPase, mutants are deficient in V-ATPase function, deletion prevents assembly of v1 domain,	(Seol et al., 2001)			
			Mutants accumulate early endosomes and have decreased endocytosis of FM4-64, mutant leads to slowed degradation but not	(Sipos et al., 2004)			

			internalization of Ste3, membrane associated protein by sedimentation assay, functions upstream of class E compartment,				
22	*YJL163C*	Putative protein of unknown function			SLC46A1 (folate transporter)	0.11	0.02
23	*Vps52*	Component of the GARP (Golgi-associated retrograde protein) complex, Vps51p-Vps52p-Vps53p-Vps54p, which is required for the recycling of proteins from endosomes to the late Golgi; involved in localization of actin and chitin	Identified in transposon mutagenesis screen for CPY secretors, vps10 and other late Golgi proteins were destabilized in deletion, requires other complex members for stability, forms 1:1:1 complex	(Conibear and Stevens, 2000)	VPS52 (Liewen et al., 2005)	0.11	0.75
			Binds ypt6	(Siniossoglou and Pelham, 2002)			
24	*VPS17*	Subunit of the membrane-associated retromer complex essential for endosome-to-Golgi retrograde protein transport; peripheral membrane protein that assembles onto the membrane with Vps5p to promote vesicle formation; required for recruiting the retromer complex to the endosome membranes	Identified in VPT screen which led to increased secretion of CPY-invertase fusion protein,	(Bankaitis et al., 1986; Robinson et al., 1988)	Snx5, Snx6 (Wassmer et al., 2009)	0.093	1
			Crosslinking experiment pulls down rest of retromer, forms a subcomplex with Vps5	(Seaman et al., 1998)			
25	*GYP7*	GTPase-activating protein for yeast Rab family members including: Ypt7p (most effective), Ypt1p, Ypt31p, and Ypt32p (in vitro); involved in vesicle mediated protein trafficking	Identified as a GAP for Ypt7 *in vitro*, Ypt7 defects not observed when Gyp7 altered (deleted or overexpressed) *in vivo*	(Vollmer et al., 1999)	TBC1D15 (Zhang et al., 2005), TBC1D17	0.083	0.01
			The GTP bound state of YPT7 promotes vacuolar fusion, Gyp7 inhibits vacuolar fusion, stimulates HOPS release	(Eitzen et al., 2000)			
26	*ECM30*	Putative protein of unknown function; may play a role in cell wall biosynthesis, mutants have abnormal relative levels of mannose and glucose and have	Transposon mutagenesis screen on calcaphlor white decreased mannose/glucose levels	(Lussier et al., 1997)	none	0.083	0.01
			Localizes to the cytoplasm	(Huh et al., 2003)			

		Gap1p sorting and transport defects; (GFP)-fusion protein localizes to the cytoplasm	Forms a complex with Ubp15 that regulates Gap1 plasma membrane localization	(Costanzo et al., 2010)			
27	*ARL1*	Soluble GTPase with a role in regulation of membrane traffic; regulates potassium influx; G protein of the Ras superfamily, similar to ADP-ribosylation factor	Identified using degenerate PCR, mutant and overexpression have growth defects, is a myristolated protein that localize to Golgi membranes,	(Lee et al., 1997)	ARL1 (Lee et al., 1997)	0.081	0.81
			Deletion is temperature-sensitive and unable to grow on sucrose suppressed by *YPT1*, increased CPY secretion	(Rosenwald et al., 2002)			
			Deletion is sensitive to hygromyocin which is suppressed by the downstream effectors, Hal4, Hal5, and Sap155, deletion does not alter Trk1	(Munson et al., 2004)			
28	*YPT6*	Rab family GTPase, Ras-like GTP binding protein involved in the secretory pathway, required for fusion of endosome-derived vesicles with the late Golgi, maturation of the vacuolar carboxypeptidase Y; has similarity to the human GTPase, Rab6	Ts mutant leads to decreased transcription of ribosomal genes, deletion is lethal at 37°C, invertase secretion and CPY glycosylation are inhibited in mutants, glycosylation pattern of invertase suggests role in ER-Golgi trafficking,	(Li and Warner, 1996)	RAB6 (Li and Warner, 1996)	0.078	0.52
			Mutants showed defects in Golgi associated glycosylation of CPY, ALP, and invertase; N-linked glycosylation enzymes are not properly localized in mutants but O-linked and ER glycosylation are, synthetic lethal with sec35 mutant, involved in Golgi/endosomal trafficking	(Luo and Gallwitz, 2003)			
29	*MVB12*	ESCRT-I subunit required to stabilize oligomers of the ESCRT-I core complex (Stp22p, Vps28p, Srn2p), which is	Colocalizes with ESCRTI by fluorescence and gel filtration, stabilizes interaction of coiled-coil domain of Vps23,	(Chu et al., 2006)	MVB12A, MVB12B (Seaman, 2008)	0.077	0.14

		involved in ubiquitin-dependent sorting of proteins into the endosome; deletion mutant is sensitive to rapamycin and nystatin	GFP tagged localizes to endosomes, deletion of ESCRTI components destabilized MVB12, Coimmunoprecipitates with vps23, deletion led to native size shift of vps23 and vps37, deletion produces a class E compartment,	(Curtiss et al., 2007)			
30	*MAK10*	Non-catalytic subunit of N-terminal acetyltransferase of the NatC type, required for replication of dsRNA virus; expression is glucose-repressible	Deletion inhibits acetlyation of NatC substrates,	(Polevoda and Sherman, 2001)	MAK10/NAA35	0.067	0.65
31	*ARL3*	GTPase of the Ras superfamily required to recruit Arl1p to the Golgi; similar to ADP-ribosylation factor	Deletion is cold-sensitive, arl3 mutants were defective in endocytosis of Lucifer Yellow, cytosolicly localized by IF and gradient analysis,	(Huang et al., 1999)	ARL3	0.065	0.85
			Acetylated by the NatC complex and recruits arl1 to the Golgi,	(Behnia et al., 2004)			
32	*RAV2*	Subunit of RAVE (Rav1p, Rav2p, Skp1p), a complex that associates with the V1 domain of the vacuolar membrane (H+)-ATPase (V-ATPase) and promotes assembly and reassembly of the holoenzyme	Associated with Skp1 and Rav1, associates with members of the Vacuolar ATPase, mutants are deficient in V-ATPase function,	(Seol et al., 2001)	none	0.064	0.13
33	*VPS13*	Protein of unknown function; heterooligomeric or homooligomeric complex; peripherally associated with membranes; involved in sporulation, vacuolar protein sorting, prospore membrane formation and protein-Golgi retention; homologous to human CHAC and COH1 which are involved in chorea acanthocytosis and Cohen syndrome, respectively	Identified in VPT screen which led to increased secretion of CPY-inveratase fusion protein, weak secretion phenotype	(Robinson et al., 1988)	VPS13A, VPS13B, VPS13C, VPS13D (Velayos-Baeza et al., 2004)	0.061	0.92
			Identified in screen that rescued mating competency of Kex2 mutant, mutants displayed normal vacuolar morphology, interferes with Kex2 retention	(Redding et al., 1996)			
			Soi1 encodes a 358kD protein that has homology to a *C. elegans* protein, immunofluorescence	(Brickner and Fuller, 1997)			

			shows altered Kex2 localization in deletion				
			Relocalizes to the prospore and is required for cytokinesis, deletion leads to decreased phosphatidylinositides at the prospore and increased intralumenal vesicles	(Park and Neiman, 2012)			
34	*TRE1*	Plasma membrane protein that binds to Bsd2p and regulates ubiquitylation and vacuolar degradation of the metal transporter Smf1p; function is redundant with that of Tre2p; has similarity to transferrin receptors	Similar to transferrin receptor, contains PY motifs, deletion with tre2 results in increased Smf1, overexpression restores growth on cadmium of *bsd2Δ*, PY motif binds Rsp5 but is not required for ubiquitination, ubiquitination depends on Bsd2	(Stimpson et al., 2006)	none	0.050	0.01
35	*COG8*	Component of the conserved oligomeric Golgi complex (Cog1p through Cog8p), a cytosolic tethering complex that functions in protein trafficking to mediate fusion of transport vesicles to Golgi compartments	Deletion causes growth defect in combination with *sec35-1*, mobility shift when deleted suggests in a complex with sec34 and sec35	(Ram et al., 2002)	COG8 (Miller and Ungar, 2012)	0.048	0.49
			Homologous to mammalian COG complex	(Ungar et al., 2002)			
			in vitro translation studies incorporation is dependent on Cog 5, Cog7, and Cog6	(Loh and Hong, 2004)			
36	*VPS30*	Subunit of phosphatidylinositol (PtdIns) 3-kinase complexes I and II; Complex I is essential in autophagy and Complex II is required for vacuolar protein sorting; ortholog of the higher eukaryotic gene Beclin 1	Identified in a morphological screen for autophagic bodies	(Tsukada and Ohsumi, 1993)	BECN1/Beclin-1 (Aita et al., 1999)	0.047	1
			Deletion secretes CPY and PrA, peripherally associates with the membrane, deletion causes mislocalization of Vps10 by gradient analysis,	(Seaman et al., 1997)			
			Identified in VPT screen which led to increased secretion of CPY-inveratase fusion protein, weak secretion phenotype	(Robinson et al., 1988)			

			Immunoprecipitation shows interaction with vps38 and vps34, deletion secretes CPY and prevents transport of API, autophagy inhibited in deletion, Vps30 binds to Vps368 which then allows binding to Apg14 and Apg15, associates with membranes,	(Kihara et al., 2001)			
			Deletion secretes CPY, deletion mislocalizes Kex2 and Vps10, deletion depletes PI3P, which inhibits Vps5 and Vps17 from binding membranes through PX domain	(Burda et al., 2002)			
			Identified in a mutagenesis screen for increased A1PiZ, UPR is constitutively active, A1PiZ is targeted to the vacuole in vps30, vps38, vps10 dependent manner for degradation	(Kruse et al., 2006)			
37	*GTR2*	Putative GTP binding protein that negatively regulates Ran/Tc4 GTPase cycle; activates transcription; subunit of EGO and GSE complexes; required for sorting of Gap1p; localizes to cytoplasm and to chromatin; homolog of human RagC and RagD	homologous to GTR1, rescues cold sensitivity of *gtr1-11* strain, GST pulldown shows interaction with GTR1 that is necessary for interaction with itself, suppresses *prp20-1*	(Nakashima et al., 1999)	RAGC, RAGD (Sekiguchi et al., 2001)	0.038	0
			Identified in screen for the inability to resume growth following rapamyocin treatment, localized to the vacuolar membrane, involved in microautphagy	(Dubouloz et al., 2005)			
			Interacts with GTR1 by yeast two hybrid, deletion decreases Gap1 activity, colocalizes with other GSE complex members on endosomal membranes, GDP bound state has higher Gap1 activity, interaction with Gap1 necessary for internalization	(Gao and Kaiser, 2006)			

38	*CCR4*	Component of the CCR4-NOT transcriptional complex, which is involved in regulation of gene expression; component of the major cytoplasmic deadenylase, which is involved in mRNA poly(A) tail shortening	Identified in a screen for regulators of ADHII activity,	(Denis, 1984)	CCR4/CNOT6 (Dupressoir et al., 2001)	0.033	0
39	*YJL132W*	Putative protein of unknown function; localizes to the membrane fraction; possible Zap1p-regulated target gene induced by zinc deficiency; YJL132W is a non-essential gene			GPLD1	0.033	0.01
40	*TCA17*	Subunit of TRAPPII, a multimeric GEF involved in intra-Golgi and endosome-to-Golgi transport; promotes association of TRAPPII-specific subunits with the core complex; sedlin related; human Sedlin mutations cause SEDT, a skeletal disorder	Identified in a screen for increased plasma membrane residence of GFP-Snc1-Suc reporter gene, Mass spec following affinity purification identified other Trapp complex members, Trs33 and Trs65 are necessary for interaction with Trs20, gel filtration shows association with TRAPPII, Involved in stability of TRS65 and Bet5 with TRAPPII, Necessary for proper localization to the Golgi	(Montpetit and Conibear, 2009)	TrappC2L (Scrivens et al., 2009)	0.033	0.01
			Interacts with the N-terminus of Trs130 by yeast two hybrid, loss of TCA17 reduces amount of Trs31 in TrappII complexes and overall reduction in TRS130	(Choi et al., 2011)			
41	*COG6*	Component of the conserved oligomeric Golgi complex (Cog1p through Cog8p), a cytosolic tethering complex that functions in protein trafficking to mediate fusion of transport vesicles to Golgi compartments	Coimmunoprecipitates with Sec35, interacts with Sec35 and Sec36 by yeast two hybrid, deletion causes growth defect similar to Sec34 and Sec35 mutants, mobility shift when deleted suggests in a complex with Sec34	(Ram et al., 2002)	COG6 ((Miller and Ungar, 2012)	0.033	0.6

			and Sec35				
			Homologous to mammalian COG complex	(Ungar et al., 2002)			
			in vitro translation studies incorporation is dependent on Cog 5 and Cog7 which is then required for incorporation of Cog8,	(Loh and Hong, 2004)			
42	*COG5*	Component of the conserved oligomeric Golgi complex (Cog1p through Cog8p), a cytosolic tethering complex that functions in protein trafficking to mediate fusion of transport vesicles to Golgi compartments	Deletion causes growth defect in combination with *sec35-1*, mobility shift when deleted suggests in a complex with Sec34 and Sec35	(Ram et al., 2002)	COG5 (Miller and Ungar, 2012)	0.031	0.35
			Homologous to mammalian COG complex	(Ungar et al., 2002)			
			in vitro translation studies show direct interaction with Cog4 and Cog7, interaction with Cog7 is required for incorporation of Cog6 which is then required for incorporation of Cog8	(Loh and Hong, 2004)			
43	*VTA1*	Multivesicular body (MVB) protein involved in endosomal protein sorting; regulates Vps4p activity by promoting its oligomerization; has an N-terminal Vps60- and Did2- binding domain, a linker region, and a C-terminal Vps4p binding domain	Interaction with Vps4 by yeast two hybrid and GST pulldown, delays post internalized degradation but not internalization of alpha factor, deletion increases CPY secretion and Ste3 accumulation, deletion forms class E compartment	(Yeo et al., 2003)	SBP1(Azmi et al., 2006)VTA1 (STRING)	0.027	0.12
			Identified in a screen for aberrant vacuolar morphology, fluid phase endocytosis delayed, CPS-GFP is mislocalized to the class E compartment in deletion, Ste3 is more stable in deletion, deletion sensitive to calcafluor white, colocalizes with Pep12,	(Shiflett et al., 2004)			

			copreciptated with Vps60				
			Decreased membrane association in vps4Δ, forms a dimer, promotes vps4 oligomerization and activation of Vps4 ATPase activity through the VSL region in the C-terminus	(Azmi et al., 2006)			
44	*OPI8*	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps verified gene DID2/YKR035W-A	Weak Opi- phenotype, overlaps *DID2*	(Hancock et al., 2006)	none	0.021	0.07
45	*TRS85*	Subunit of TRAPPIII (transport protein particle), a multimeric guanine nucleotide-exchange factor for Ypt1p, required for membrane expansion during autophagy and the CVT pathway; directs Ypt1p to the PAS; late post-replication meiotic role	Pulled down by Bet3-PrA with TRAPP complex, overexpression suppresses *bet3-1*	(Sacher et al., 2000)	none	0.019	0.41
			Identified in a screen for nitrogen starvation sensitivity, deletion leads to reduced autophagy and pexophagy, involved in CVT vesicle formation	(Meiling-Wesse et al., 2005)			
			Not a part of TRAPPII but is a part of TRAPPI	(Choi et al., 2011)			
46	*SAP155*	Protein required for function of the Sit4p protein phosphatase; forms a complex with Sit4p; member of a family of similar proteins including Sap4p, Sap185p, and Sap190p; protein abundance increases in response to DNA replication stress	Coimmunoprecipitates with Sit4-HA and Sit4:PY,overexpression suppresses temperature sensitivity of *sit4-102*, deletion causes slowed growth on YPD, function of Sit4 requires Saps	(Luke et al., 1996)	PP6R1, PP6R2, PP6R3 (Stefansson and Brautigan, 2006)	0.015	0.01
47	*COG7*	Component of the conserved oligomeric Golgi complex (Cog1p through Cog8p), a cytosolic tethering	Deletion causes growth defect in combination with *sec35-1*, mobility shift when deleted suggests in a complex	(Ram et al., 2002)	COG7 (Miller and Ungar, 2012)	0.014	0.38

		complex that functions in protein trafficking to mediate fusion of transport vesicles to Golgi compartments	with Sec34 and Sec35				
			Homologous to mammalian COG complex	(Ungar et al., 2002)			
			in vitro translation studies show direct interaction with Cog4 and Cog5, interaction with Cog5 is required for incorporation of Cog6 which is then required for incorporation of Cog8	(Loh and Hong, 2004)			
48	*GDH3*	NADP(+)-dependent glutamate dehydrogenase; synthesizes glutamate from ammonia and alpha-ketoglutarate; rate of alpha-ketoglutarate utilization differs from Gdh1p; expression regulated by nitrogen and carbon sources; GDH3 has a paralog, GDH1, that arose from the whole genome duplication			GLUD1, GLUD2 (STRING)	0.013	0
49	*YJL118W*	Putative protein of unknown function; may interact with ribosomes, based on co-purification experiments; YJL18W is a non-essential gene; deletion enhances the toxicity of heterologously expressed human alpha-synuclein			None	0.0097	0
50	*VPS53*	Component of the GARP (Golgi-associated retrograde protein) complex, Vps51p-Vps52p-Vps53p-Vps54p, which is required for the recycling of proteins from endosomes to the late Golgi; required for vacuolar protein sorting	Identified in transposon mutagenesis screen for CPY secretors, vps10 and other late Golgi proteins were destabilized in deletion, requires other complex members for stability, forms 1:1:1 complex	(Conibear and Stevens, 2000)	Vps53 (Liewen et al., 2005)	0.0095	0.01

51	*IRS4*	EH domain-containing protein involved in regulating phosphatidylinositol 4,5-bisphosphate levels and autophagy; Irs4p and Tax4p bind and activate the PtdIns phosphatase Inp51p; Irs4p and Tax4p are involved in localizing Atg17p to the PAS			TRIOBP (STRING)	0.0079	0.52
52	*RGD2*	GTPase-activating protein (RhoGAP) for Cdc42p and Rho5p	GAP identified *in silico*, binding confirmed by yeast 2 hybird, activity measured by the MESG/phosphorylase *in vitro* assay	(Roumanie et al., 2001)	none	0.0074	0.02
53	*BGL2*	Endo-beta-1,3-glucanase, major protein of the cell wall, involved in cell wall maintenance	Purified protein used to identify gene, cloned gene had homology to beta1,3 exoglucanase, disruption did not affect transformation, growth, osmotic stability, mating, and sporulation	(Klebl and Tanner, 1989)	none	0.0045	0.03
			Beta1,3 endoglucanse not exoglucanse, enzyme characterized, overproduction causes slow growth phenotype	(Mrsa et al., 1993)			
			Hypoosmotic screen revealed overproduciton of BGL2, phenotype complemented by PKC1	(Shimizu et al., 1994)			
			Factor of *c. albicans* virulence	(Sarthy et al., 1997)			
			Deletion of homolog in *Aspergillus fumigatus* showed no phenotype	(Mouyna et al., 1998)			
	WT					0	0

[a] Descriptions from the Saccharomyces Genome Database

[b] Summaries from the literature of select genes involved in trafficking, localization, and stability

[c] Growth Score calculated from doubling times (see Materials and Methods)

[d] CPY secretion score (Bonangelino et al., 2002)

APPENDIX B

PROTEIN QUALITY CONTROL OF THE EPITHELIAL SODIUM CHANNEL

(Sections of this Appendix were previously published in Buck, T.M., A.R. Kolb, C.R. Boyd, T.R. Kleyman, and J.L. Brodsky. 2010. The endoplasmic reticulum-associated degradation of the epithelial sodium channel requires a unique complement of molecular chaperones. *Mol Biol Cell*. 21:1047-58.)

B.1 INTRODUCTION

ENaC represents another ion channel that can be used in yeast studies to reveal protein quality control mechanisms. ENaC is a sodium channel composed of three homologous subunits (α, β, γ) localized to the kidney, airway, and colon epithelia that functions to maintain sodium and volume homeostasis (Snyder, 2005; Soundararajan et al., 2010). Misregulation of ENaC surface expression leads to diseases that alter blood pressure such as Liddle's syndrome and Pseudohypoaldosteronism (Snyder et al., 2002; Su and Menon, 2001). ENaC has also been shown to interact with the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) (Berdiev et al., 2009) and has been implicated in Cystic Fibrosis phenotypes (Gentzsch et al., 2010; Huber et al., 2010; Rauh et al., 2010). Thus, ENaC levels and function must be tightly regulated throughout the secretory pathway. It is somewhat surprising that studies have focused

almost exclusively on the later steps of regulation even though less than 20% of the protein ultimately resides in the membrane (Hanwell et al., 2002; Rotin et al., 2001; Valentijn et al., 1998).

In this appendix, I describe my work aimed at understanding how ENaC is subject to protein quality control in the ER. I first characterize the ubiquitination requirements of the ENaC subunits during ERAD in yeast. I then describe my attempts to establish the conditions for a screen of ENaC plasma membrane regulators.

B.2 UBIQUITINATION OF ENAC

The ENaC subunits are ERAD substrates, and Dr. Buck demonstrated that ENaC ERAD is dependent on both Hrd1 and Doa10 in yeast. Since these ligases are typically involved in recognizing different types of lesions, Hrd1 and Doa10 may be performing an additional function besides ubiquitination of the substrate, such as retrotranslocation of the substrate (Carvalho et al., 2010; Zhong and Fang, 2012). In addition, the lumenal Hsp40's, Scj1 and Jem1 are required for ENaC degradation, but unlike other ERAD substrates, the Hsp70, Kar2 was not required. Thus, ENaC likely requires a unique set of protein quality control factors. However, because these chaperones appear to be acting in a unique way, it is not clear at what step during ERAD they are required. To address these questions, I examined the ubiquitination of ENaC using an *in vitro* and an *in vivo* assay.

B.2.1 Materials and Methods - Ubiquitination Assays

The *in vitro* ubiquitination assay (Nakatsukasa *et al.,* 2008) was adapted to measure the extent of ubiquitinated ENaC in ER-derived microsomes prepared from wild-type and mutant yeast strains expressing the indicated HA-tagged ENaC subunit. Microsomes and cytosol from transformed strains grown in selective medium were prepared as described (McCracken and Brodsky, 1996), and 20 µl ubiquitination reactions contained ~20 µg of microsomes, an ATP-regenerating system, and 2 mg/ml cytosol in B88 (20 mM HEPES, pH 6.8, 150 mM KOAc, 250 mM sorbitol, 5 mM MgOAc, and a protease inhibitor cocktail [2 µg/ml leupeptin, 2 µg/ml aprotinin, 10 µg/ml trypsin inhibitor, 1 µg/ml E64, 10 µg/ml TPCK, 1 mM PMSF, 0.1 µg/ml pepstatin A]). Reactions were assembled on ice with protease inhibitor cocktail and were then prewarmed to 23°C for 10 min before 2 µl of 125 I-labeled ubiquitin was added (~200,000 cpm/µl). At the indicated time point, the reaction was quenched with 1.25% SDS containing a protease inhibitor cocktail and 10 mM N-ethylmaleimide (NEM). ENaC subunits were immunoprecipitated with anti-HA conjugated resin (Roche) with protease inhibitors. The precipitated samples were split and half of the material was used for a Western blot analysis to detect the precipitated ENaC subunit and the other half was used for phosphorimager analysis. Data were analyzed and quantified using Image Gauge Software (v3.45; Fuji Film Science Lab).

The detection of ubiquitinated ENaC in yeast was performed essentially as described (Ahner et al., 2007). In brief, cells expressing the indicated HA-tagged ENaC subunit were grown to log phase in 30 ml of selective medium. Next, the cells were disrupted with glass beads in lysis buffer (150 mM NaCl, 50 mM Tris, pH7.5, 0.1% NP40, 10 mM NEM, and a protease inhibitor cocktail) by agitation on a Vortex mixer 10 times for 30 s with 30 s incubations on ice between each cycle. Cell debris was removed by centrifugation, and the protein concentration

was estimated by measuring the absorbance at 280 nm. ENaC was immunoprecipitated from equal amounts of lysate with anti-HA–conjugated resin (Roche), and the precipitated proteins were resolved by SDS-PAGE. Half of each sample was used to detect ENaC, and the other half was used to detect polyubiquitinated ENaC. ENaC was detected with anti-HA antibody (Roche), and ubiquitinated ENaC was detected with anti-ubiquitin antiserum (obtained from the laboratory of C. Pickart, Johns Hopkins University School of Medicine [deceased]) after boiling the nitrocellulose membrane for 30 min in water. Data were obtained and quantified as described above.

B.2.2 Results – Ubiquitination Assays

Dr. Buck in the Brodsky lab demonstrated that ENaC degradation is dependent on both Hrd1 and Doa10. When either of these is deleted there is significant stabilization of the ENaC subunits, and when both are deleted, there is synergistic stabilization. To assay whether the extent of stabilization correlated with the degree of ubiquitination—and thus that the effect of deleting the ligases was direct—I examined α- and β-subunit ubiquitination *in vivo*. To this end, the α- and β-subunits were immunoprecipitated from wild type and from *hrd1*Δ and *hrd1*Δ*doa10*Δ mutant yeast, and an anti-ubiquitin antibody was used in a subsequent Western blot analysis. The results presented in Figure 16A and B, indicate that subunit ubiquitination was significantly decreased in yeast lacking Hrd1 or both Hrd1 and Doa10. These data are consistent with a requirement for these ligases during ERAD, as observed (Buck et al., 2010), and indicate that the effects of deleting the ligases are direct.

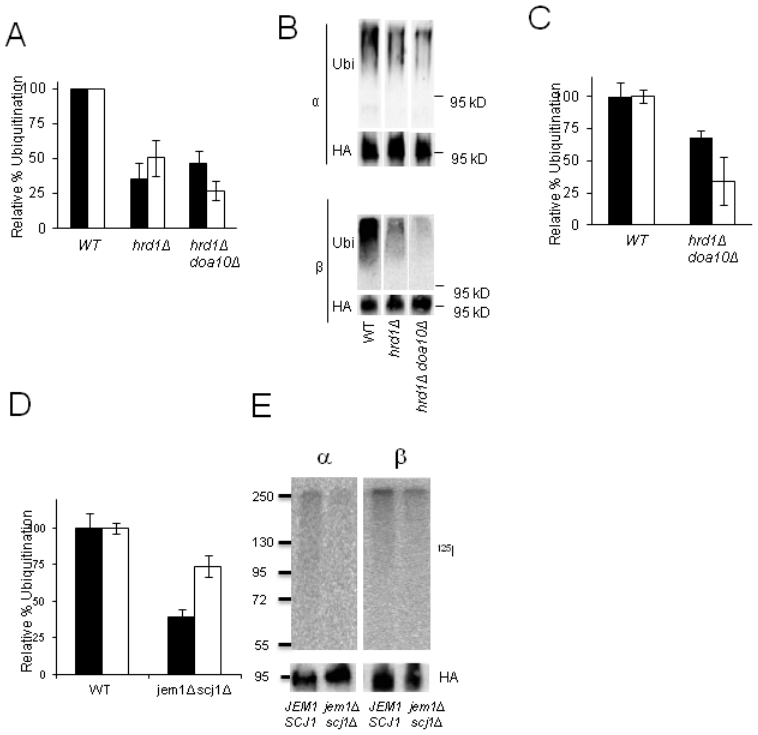


Figure 16: Ubiquitination of ENaC

ENaC ubiquitination is impaired in yeast strains lacking the E3 ubiquitin ligases Hrd1 and Doa10. A) Wild type (WT) or the indicated mutant yeast strains expressing either the α- (■) or β- (□) ENaC subunit were processed as described and the level of ubiquitination was assessed. The bar graph represents the means of 5–7 determinations, ±SEM, and the data in the mutant strains were standardized to the amount in the wild-type cells. A typical experimental result is shown in B. C) Microsomes from wild type (*HRD1/DOA10*) or the *hrd1Δ doa10Δ* mutant strain expressing either α- or β-ENaC were prepared and subjected to the *in vitro* ubiquitination assay. The bar graphs represent the means of three (β) or eight (α) determinations, ±SEM. D) Microsomes from wild type (*JEM1/SCJ1*) or the *jem1Δ scj1Δ* mutant strain expressing either α- or β-ENaC were prepared and subjected to the in vitro ubiquitination assay as described. The bar graphs represent the means of six determinations, ±SEM. A typical result is shown in E.

To confirm these data, I next developed an assay in which the degree of ENaC subunit ubiquitination could be assessed *in vitro*. This assay was based on a system in which the conjugation of ^{125}I-ubiquitin onto membrane-integrated ERAD substrates could be monitored after their expression in yeast (Nakatsukasa et al., 2008). I therefore prepared ER-derived microsomes from α-ENaC-expressing yeast and delineated the time, temperature, and cytosol dependence on ENaC subunit ubiquitination *in vitro* (Figure 17). I found that the optimal ENaC ubiquitination was observed when the reactions were allowed to proceed for 40 min. with 1-2mg/mL of cytosol.

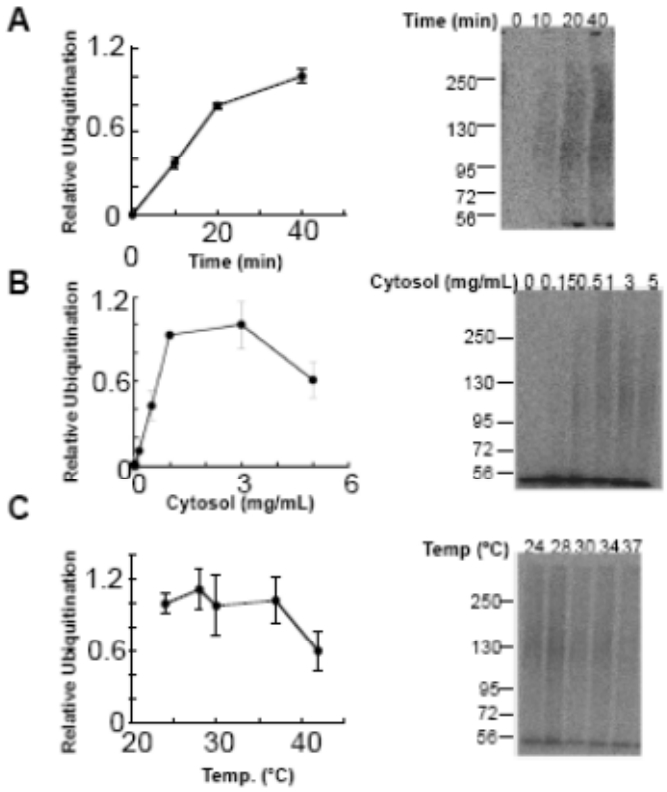


Figure 17: Optimization of the in vitro ubiquitination assay for α-ENaC.

Microsomes were prepared from yeast expressing α-ENaC and the *in vitro* ubiquitination assay was performed as described under the indicated conditions. In **A** reactions contained 2 mg/mL of cytosol and were performed at 23°C, in **B** reactions were performed at 23°C for 40 min, and in **C** reactions contained 2 mg/mL cytosol and were performed for 40 min.

I next prepared microsomes from either wild-type yeast or *hrd1*Δ*doa10*Δ cells that expressed the α- or β-subunit and used the optimized conditions to assess subunit ubiquitination *in vitro*. I again found that subunit ubiquitination decreased in the ligase mutant strain (Figure 16C). These results suggest that Hrd1 and Doa10 ubiquitinate ENaC directly.

Further, Dr Buck identified the Hsp40s, Scj1 and Jem1, as another set of factors that participate in the ERAD of ENaC. ENaC degradation was stabilized when *SCJ1* and *JEM1* were deleted. The inefficient degradation of the ENaC subunits in the *scj1*Δ *jem1*Δ strain might either result from an inability of the protein to be delivered or recognized by E3 ligases or result from an inability to transfer the ubiquitinated species to the proteasome. To differentiate between these scenarios, I assessed subunit ubiquitination *in vitro*, as described above, in a wild-type strain and in cells lacking Scj1 and Jem1 (Figure 16D, E). The relative percentage of ubiquitinated protein decreased significantly for both the α- and β-subunit, consistent with the Hsp40s playing a role before ubiquitination in the ERAD process.

B.3 A YEAST SCREEN TO IDENTIFY EFFECTORS OF ENAC PLASMA MEMBRANE RESIDENCE

ENaC is an ERAD substrate that functions at the plasma membrane and like Kir2.1 it must assemble into a multimeric channel, although in the case of ENaC it is a trimer (Stewart et al., 2011; Stockand et al., 2008). The most active channel consists of α, β, and γ subunits. Elimination of either β or γ ENaC reduces current to 10% of the full channel activity, and α alone produces only ~1% of the activity (Canessa et al., 1994b). Although ENaC is normally highly selective for sodium over potassium (1000 Na^+:1 K^+), a mutation can be made within the α-ENaC selectivity filter to make it more permissive to potassium over sodium ($4K^+$: $1Na^+$) (Sheng et al., 2000). While this mutation favors potassium, it still is not as selective as a natural potassium channel, such as Kir2.1, a potassium channel expressed in skeletal muscle, heart, brain, lung, and kidney (Kubo et al., 1993; Tang et al., 1995a). I attempted to establish a screen for α-ENaC similar to that described in Chapter 2. To this end I attempted to find conditions in which when the potassium permissive ENaC mutant was expressed in a *trk1Δ trk2Δ* strain it would rescue growth on low potassium.

B.3.1 Attempts to optimize a screen for alpha ENaC

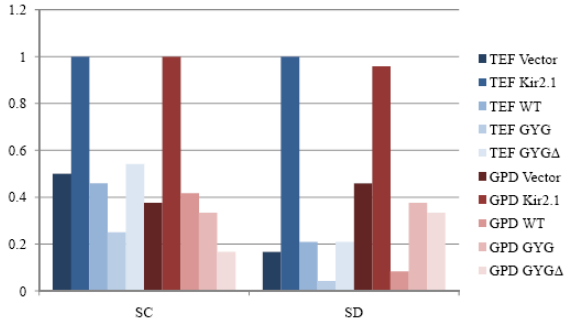
To find the optimal conditions to use for the screen, I made a series of wild type and mutant ENaC expression plasmids to test on various media. The mutant ENaC constructs include the

selectivity filter mutant ^{587}GYG^{589}, and the GYG mutant with a 26 amino acid fragment cleaved that has been shown to increase channel open probability (GYGΔ) (Carattino et al., 2007; Sheng et al., 2000). To regulate ENaC expression, I placed these constructs under the control of different promoters: *GPD* (highest constitutive expression), *TEF* (high constitutive expression), *MET25* (methionine repressible), and *GAL* (galactose inducible). Dr Buck previously made the *GPD* plasmids. To construct the remaining plasmids, I used the backbone of PRS4x6 vectors (Mumberg et al., 1995) and inserted the ENaC construct into the polylinker by cutting the construct from the *GPD* vectors with EcoR1 and Xho1. All plasmids were made and confirmed by sequencing. The plasmids were transformed into yeast lacking Trk1 and Trk2 and tested for rescue of growth under media conditions that varied for potassium concentration (100, 50, 30, 0mM K+ added), pH (3, 4, 6), and amino acid supplement (SC, SD). For these efforts I was assisted by Cary Boyd, who was a postdoctoral PSTP researcher.

Expression of Kir2.1 always worked as a positive control, however sometimes the vector control would appear to rescue, most likely due to the presence of suppressors. The potassium selective mutant also gave inconsistent results (Figure 18). This inconsistency was seen regardless of the plasmid or media conditions and may be due to suppressors (Liang et al., 1998; Vidal et al., 1990; Wright et al., 1996) or differences in plasmid copy number. Even if the results were averaged a condition and ENaC construct combination could not be found that produced a signal detectable over background. Although there were times when the ENaC mutants rescued as predicted, there was not enough consistency to do a reliable screen. Nevertheless some trends could be seen. All ENaC constructs, regardless of promoter, grew at pH 6 even at the lowest potassium concentration, while growth became more selective at lower pH. In addition growth was slower on SD media and at lower pH. There also did not appear to

be a difference between the GYG and the GYGΔ construct. Thus, the GYGΔ was not considered for future studies.

A)



B)

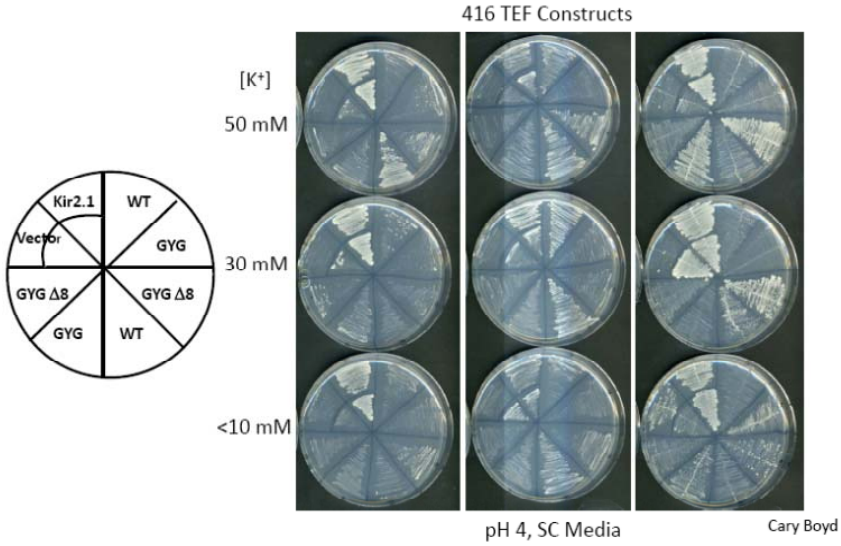


Figure 18: Growth assays of *trk1Δ trk2Δ* yeast expressing ENaC mutants

A) Summary of all experiments done with either the PRS416 TEF vectors or the GPD 426 vectors. Each experiment was scored as a 1 for growth or 0 for no growth and the total was divided by the number of experiments. B) *trk1Δ trk2Δ* strains containing PRS416 TEF plasmids with either Kir2.1, α-ENaC (WT), α-ENaC with ^{587}GYG^{589} mutation (GYG), or ^{587}GYG^{589} mutation with the inhibitory peptide deleted (GYGΔ) were streaked onto –URA plates supplemented with 50, 30 or 0 mM K^+ at a pH of 4. Shown are three separate experiments indicating the inconsistency of the results.

To overcome issues with plasmid copy number and provide a more consistent context for the screen, I integrated the *TEF*, *MET25*, and *GAL* constructs into the *TRK1* locus of a yeast strain already deleted for *TRK2* with the *NAT* resistance gene. I designed primers to an area in the backbone common to all the plasmids used that flanked the *URA* gene and ENaC. This ~5.5kb region was PCR amplified and transformed into yeast and allowed to integrate by homologous recombination. Integration was confirmed by PCR using one internal primer to the PCR amplified region and one external primer to the *TRK1* locus. The resulting integrants were then tested in similar conditions as above. I confirmed and tested all of the *MET25* constructs.

None of the *MET25* constructs rescued growth on low potassium, including the positive control, Kir2.1. One possibility for failed rescue may be due to insufficient protein expression levels. To confirm this, a time course performed by Cary Boyd showed that expression of ENaC ceased 20hrs after the removal of methionine in liquid culture. As a way to further increase channel activity, and thus its ability to augment growth on low potassium, I expressed α-ENaC with either β- or γ-ENaC. However, this caused the strain to grow so slowly that a growth screen was infeasible.

As an alternative approach I attempted to perform a sodium sensitivity screen with wild type ENaC. To this end I engineered a yeast strain that lacked the ENA genes that encode the primary sodium efflux system (Garciadeblas et al., 1993; Haro et al., 1991). Yeast encode up to 4 copies of this gene, depending on the strain. I deleted ENA by homologous recombination and the resulting strain was sensitive to sodium. However, ENaC could not be expressed in this strain, and I could not identify conditions that would allow for a screen for ENaC protein quality control factors. Instead, I performed the Kir2.1 screen described in chapter 2.

B.3.2 Does ENaC function at the plasma membrane in yeast

One possibility why a screen using ENaC was not successful is that there was not enough functional channel at the plasma membrane. Like mammalian cells the majority of ENaC localizes to the ER in yeast (Buck et al., 2010). Only a small population (less than 5%) traffics to the plasma membrane (Figure 19 lanes 17-19) and this may not be enough to produce a noticeable phenotype. A plasma membrane population of ENaC would be consistent with previous results that showed that expression of ENaC made cells sensitive to high concentrations of sodium (Gupta and Canessa, 2000). However, it is not clear whether the observed toxicity is due only to excessive sodium entering the cell through the channel or whether overexpressing a heterologous protein also contributed to the increased sensitivity of the yeast. In fact, yeast expressing ENaC grow more slowly and my analysis of the unfolded protein response using a β-galactosidase reporter showed that ENaC expression highly induces the UPR, suggesting that it is the expression and not the function of ENaC that mediates toxicity (Table 5). Thus, ENaC may not be suited for a screen that depends on it functioning at the plasma membrane.

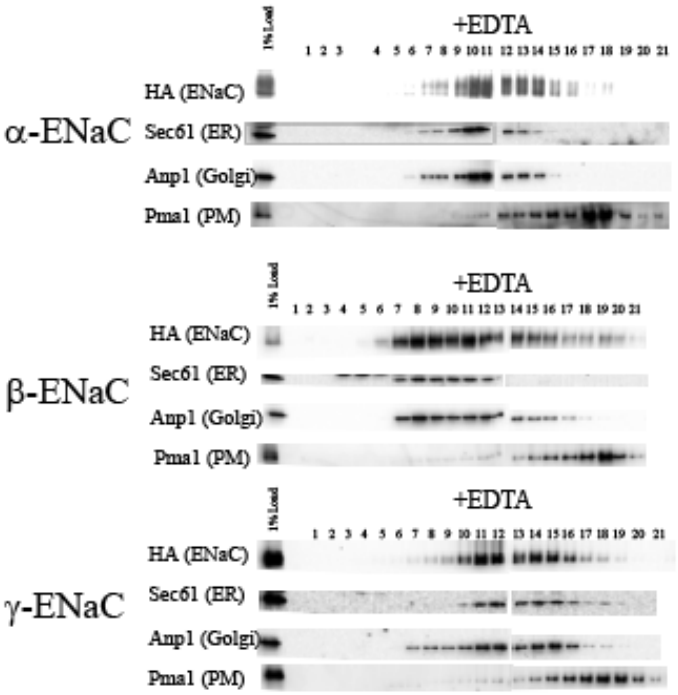


Figure 19: Sucrose gradient analysis of ENaC

The ENaC subunits were individually expressed in yeast and subjected to sucrose gradient analysis as described in chapter 2. Cell lysates prepared from yeast expressing the ENaC subunits were centrifuged on a 20-70% sucrose gradient containing either EDTA or Mg^{2+}. Gradients were performed in the absence of Mg^{2+} (+EDTA) to separate plasma membrane from Golgi and ER and in the presence of Mg^{2+} to separate Golgi from PM and ER. Fractions were taken, TCA precipitated and immunoblotted for HA (ENaC), Sec61 (ER marker), Anp1 (Golgi marker), and Pma1 (plasma membrane marker).

Table 6: The UPR is induced in yeast strains expressing ENaC.

Strain/Protein		WT/pRS326	WT/α-ENaC	WT/β-ENaC	*ire1Δ*/pRS326
UPR	(-DTT)	600	4000	2700	5.0
	(+DTT)	1500	3700	3100	7.8

The UPR (in β-galactosidase units) in the indicated strains was measured from cells that were grown in the presence or absence of 5 mM DTT for 1 h as described (Kabani et al., 2003). PRS326 is a vector control that lacks an insert.

BIBLIOGRAPHY

(2010). Database of Interacting Proteins [online]. UCLA. http://dip.doe-mbi.ucla.edu/dip/Main.cgi [2011].

(2011) BioGRID [online]. TyersLab.com. http://thebiogrid.org. [2011].

Saccharomyces Genome Database [online]. Stanford University. http://www.yeastgenome.org. [2011].

USRDS 2009 Annual Data Report. United States Renal Data System Web site [online]. www.usrds.org/adr.htm. (The USRDS End-Stage Renal Disease Incident and Prevalent Quarterly Update is available at www.usrds.org/qtr/qrt_report_table_c_ Q1_09.html.) (2011).

Yeast GFP Fusion Localization Database. http://yeastgfp.yeastgenome.org/.

Adams, A., Kaiser C., and Cold Spring Harbor Laboratory. 1998. Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual. Cold Spring Harbor Laboratory Press, Plainview, NY.

Aebi, M., R. Bernasconi, S. Clerc, and M. Molinari. 2010. N-glycan structures: recognition and processing in the ER. *Trends Biochem Sci*. 35:74-82.

Ahner, A., K. Nakatsukasa, H. Zhang, R.A. Frizzell, and J.L. Brodsky. 2007. Small heat-shock proteins select deltaF508-CFTR for endoplasmic reticulum-associated degradation. *Mol Biol Cell*. 18:806-14.

Aita, V.M., X.H. Liang, V.V. Murty, D.L. Pincus, W. Yu, E. Cayanis, S. Kalachikov, T.C. Gilliam, and B. Levine. 1999. Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21. *Genomics*. 59:59-65.

Alderson, N.L., B.M. Rembiesa, M.D. Walla, A. Bielawska, J. Bielawski, and H. Hama. 2004. The human FA2H gene encodes a fatty acid 2-hydroxylase. *J Biol Chem*. 279:48562-8.

Alesutan, I., C. Munoz, M. Sopjani, M. Dermaku-Sopjani, D. Michael, S. Fraser, B.E. Kemp, G. Seebohm, M. Foller, and F. Lang. 2011. Inhibition of Kir2.1 (KCNJ2) by the AMP-activated protein kinase. *Biochem Biophys Res Commun*. 408:505-10.

Amerik, A.Y., J. Nowak, S. Swaminathan, and M. Hochstrasser. 2000. The Doa4 deubiquitinating enzyme is functionally linked to the vacuolar protein-sorting and endocytic pathways. *Mol Biol Cell*. 11:3365-80.

Apaja, P.M., H. Xu, and G.L. Lukacs. 2010. Quality control for unfolded proteins at the plasma membrane. *J Cell Biol*. 191:553-70.

Araki, K., and K. Nagata. 2011. Protein folding and quality control in the ER. *Cold Spring Harb Perspect Biol*. 3:a007526.

Arias, E., and A.M. Cuervo. 2011. Chaperone-mediated autophagy in protein quality control. *Curr Opin Cell Biol*. 23:184-9.

Arino, J., J. Ramos, and H. Sychrova. 2010. Alkali metal cation transport and homeostasis in yeasts. *Microbiol Mol Biol Rev*. 74:95-120.

Armstrong, J., S. Patel, and P. Riddle. 1990. Lysosomal sorting mutants of coronavirus E1 protein, a Golgi membrane protein. *J Cell Sci*. 95 (Pt 2):191-7.

Arteaga, M.F., L. Wang, T. Ravid, M. Hochstrasser, and C.M. Canessa. 2006. An amphipathic helix targets serum and glucocorticoid-induced kinase 1 to the endoplasmic reticulum-associated ubiquitin-conjugation machinery. *Proc Natl Acad Sci U S A*. 103:11178-83.

Arvan, P., X. Zhao, J. Ramos-Castaneda, and A. Chang. 2002. Secretory pathway quality control operating in Golgi, plasmalemmal, and endosomal systems. *Traffic*. 3:771-80.

Ashok, A., and R.S. Hegde. 2009. Selective processing and metabolism of disease-causing mutant prion proteins. *PLoS Pathog*. 5:e1000479.

Aubin-Tam, M.E., A.O. Olivares, R.T. Sauer, T.A. Baker, and M.J. Lang. Single-molecule protein unfolding and translocation by an ATP-fueled proteolytic machine. *Cell*. 145:257-67.

Azmi, I., B. Davies, C. Dimaano, J. Payne, D. Eckert, M. Babst, and D.J. Katzmann. 2006. Recycling of ESCRTs by the AAA-ATPase Vps4 is regulated by a conserved VSL region in Vta1. *J Cell Biol*. 172:705-17.

Azmi, I.F., B.A. Davies, J. Xiao, M. Babst, Z. Xu, and D.J. Katzmann. 2008. ESCRT-III family members stimulate Vps4 ATPase activity directly or via Vta1. *Dev Cell*. 14:50-61.

Babst, M., D.J. Katzmann, E.J. Estepa-Sabal, T. Meerloo, and S.D. Emr. 2002a. Escrt-III: an endosome-associated heterooligomeric protein complex required for mvb sorting. *Dev Cell*. 3:271-82.

Babst, M., D.J. Katzmann, W.B. Snyder, B. Wendland, and S.D. Emr. 2002b. Endosome-associated complex, ESCRT-II, recruits transport machinery for protein sorting at the multivesicular body. *Dev Cell*. 3:283-9.

Babst, M., G. Odorizzi, E.J. Estepa, and S.D. Emr. 2000. Mammalian tumor susceptibility gene 101 (TSG101) and the yeast homologue, Vps23p, both function in late endosomal trafficking. *Traffic*. 1:248-58.

Babst, M., T.K. Sato, L.M. Banta, and S.D. Emr. 1997. Endosomal transport function in yeast requires a novel AAA-type ATPase, Vps4p. *EMBO J*. 16:1820-31.

Babst, M., B. Wendland, E.J. Estepa, and S.D. Emr. 1998. The Vps4p AAA ATPase regulates membrane association of a Vps protein complex required for normal endosome function. *EMBO J*. 17:2982-93.

Baietti, M.F., Z. Zhang, E. Mortier, A. Melchior, G. Degeest, A. Geeraerts, Y. Ivarsson, F. Depoortere, C. Coomans, E. Vermeiren, P. Zimmermann, and G. David. 2012. Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. *Nat Cell Biol*. 14:677-85.

Bankaitis, V.A., L.M. Johnson, and S.D. Emr. 1986. Isolation of yeast mutants defective in protein targeting to the vacuole. *Proc Natl Acad Sci U S A*. 83:9075-9.

Barreto, L., D. Canadell, S. Petrezselyova, C. Navarrete, L. Maresova, J. Perez-Valle, R. Herrera, I. Olier, J. Giraldo, H. Sychrova, L. Yenush, J. Ramos, and J. Arino. 2011. A genomewide screen for tolerance to cationic drugs reveals genes important for potassium homeostasis in Saccharomyces cerevisiae. *Eukaryot Cell*. 10:1241-50.

Bayascas, J.R. 2008. Dissecting the role of the 3-phosphoinositide-dependent protein kinase-1 (PDK1) signalling pathways. *Cell Cycle*. 7:2978-82.

Bays, N.W., and R.Y. Hampton. 2002. Cdc48-Ufd1-Npl4: stuck in the middle with Ub. *Curr Biol*. 12:R366-71.

Becker, J., W. Walter, W. Yan, and E.A. Craig. 1996. Functional interaction of cytosolic hsp70 and a DnaJ-related protein, Ydj1p, in protein translocation in vivo. *Mol Cell Biol*. 16:4378-86.

Beguin, P., U. Hasler, O. Staub, and K. Geering. 2000. Endoplasmic reticulum quality control of oligomeric membrane proteins: topogenic determinants involved in the degradation of the

unassembled Na,K-ATPase alpha subunit and in its stabilization by beta subunit assembly. *Mol Biol Cell*. 11:1657-72.

Behnia, R., B. Panic, J.R. Whyte, and S. Munro. 2004. Targeting of the Arf-like GTPase Arl3p to the Golgi requires N-terminal acetylation and the membrane protein Sys1p. *Nat Cell Biol*. 6:405-13.

Beitz, E., B. Wu, L.M. Holm, J.E. Schultz, and T. Zeuthen. 2006. Point mutations in the aromatic/arginine region in aquaporin 1 allow passage of urea, glycerol, ammonia, and protons. *Proc Natl Acad Sci U S A*. 103:269-74.

Belcher, C.N., and N. Vij. 2010. Protein processing and inflammatory signaling in Cystic Fibrosis: challenges and therapeutic strategies. *Curr Mol Med*. 10:82-94.

Benedetti, H., S. Raths, F. Crausaz, and H. Riezman. 1994. The END3 gene encodes a protein that is required for the internalization step of endocytosis and for actin cytoskeleton organization in yeast. *Mol Biol Cell*. 5:1023-37.

Berdiev, B.K., Y.J. Qadri, and D.J. Benos. 2009. Assessment of the CFTR and ENaC association. *Mol Biosyst*. 5:123-7.

Bernhardt, F., W. Schoner, B. Schroeder, G. Breves, and G. Scheiner-Bobis. 1999. Functional expression and characterization of the wild-type mammalian renal cortex sodium/phosphate cotransporter and an 215R mutant in Saccharomyces cerevisiae. *Biochemistry*. 38:13551-9.

Biber, J., N. Hernando, I. Forster, and H. Murer. 2009. Regulation of phosphate transport in proximal tubules. *Pflugers Arch*. 458:39-52.

Bienert, G.P., A.L. Moller, K.A. Kristiansen, A. Schulz, I.M. Moller, J.K. Schjoerring, and T.P. Jahn. 2007. Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. *J Biol Chem*. 282:1183-92.

Bilodeau, P.S., J.L. Urbanowski, S.C. Winistorfer, and R.C. Piper. 2002. The Vps27p Hse1p complex binds ubiquitin and mediates endosomal protein sorting. *Nat Cell Biol*. 4:534-9.

Blond-Elguindi, S., S.E. Cwirla, W.J. Dower, R.J. Lipshutz, S.R. Sprang, J.F. Sambrook, and M.J. Gething. 1993. Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. *Cell*. 75:717-28.

Bobulescu, I.A., and O.W. Moe. 2009. Luminal Na(+)/H (+) exchange in the proximal tubule. *Pflugers Arch*. 458:5-21.

Bockenhauer, D., S. Feather, H.C. Stanescu, S. Bandulik, A.A. Zdebik, M. Reichold, J. Tobin, E. Lieberer, C. Sterner, G. Landoure, R. Arora, T. Sirimanna, D. Thompson, J.H. Cross, W. van't Hoff, O. Al Masri, K. Tullus, S. Yeung, Y. Anikster, E. Klootwijk, M. Hubank, M.J. Dillon, D. Heitzmann, M. Arcos-Burgos, M.A. Knepper, A. Dobbie, W.A. Gahl, R. Warth, E. Sheridan, and R. Kleta. 2009. Epilepsy, ataxia, sensorineural deafness, tubulopathy, and KCNJ10 mutations. *N Engl J Med*. 360:1960-70.

Bonangelino, C.J., E.M. Chavez, and J.S. Bonifacino. 2002. Genomic screen for vacuolar protein sorting genes in Saccharomyces cerevisiae. *Mol Biol Cell*. 13:2486-501.

Bordallo, J., R.K. Plemper, A. Finger, and D.H. Wolf. 1998. Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. *Mol Biol Cell*. 9:209-22.

Boulis, N.M., C.R. Handy, C.A. Krudy, E.M. Donnelly, T. Federici, C.K. Franz, E.M. Barrow, Q. Teng, P. Kumar, and D. Cress. 2013. Regulated Neuronal Neuromodulation via Spinal Cord Expression of the Gene for the Inwardly Rectifying Potassium Channel 2.1 (Kir2.1). *Neurosurgery*.

Brachmann, C.B., A. Davies, G.J. Cost, E. Caputo, J. Li, P. Hieter, and J.D. Boeke. 1998. Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast*. 14:115-32.

Breslow, D.K., D.M. Cameron, S.R. Collins, M. Schuldiner, J. Stewart-Ornstein, H.W. Newman, S. Braun, H.D. Madhani, N.J. Krogan, and J.S. Weissman. 2008. A comprehensive strategy enabling high-resolution functional analysis of the yeast genome. *Nat Methods*. 5:711-8.

Brew, C.T., and T.C. Huffaker. 2002. The yeast ubiquitin protease, Ubp3p, promotes protein stability. *Genetics*. 162:1079-89.

Brickner, J.H., and R.S. Fuller. 1997. SOI1 encodes a novel, conserved protein that promotes TGN-endosomal cycling of Kex2p and other membrane proteins by modulating the function of two TGN localization signals. *J Cell Biol*. 139:23-36.

Brodsky, J.L., and R. Schekman. 1993. A Sec63p-BiP complex from yeast is required for protein translocation in a reconstituted proteoliposome. *J Cell Biol*. 123:1355-63.

Brodsky, J.L., E.D. Werner, M.E. Dubas, J.L. Goeckeler, K.B. Kruse, and A.A. McCracken. 1999. The requirement for molecular chaperones during endoplasmic reticulum-associated protein degradation demonstrates that protein export and import are mechanistically distinct. *J Biol Chem*. 274:3453-60.

Buck, T.M., A.R. Kolb, C.R. Boyd, T.R. Kleyman, and J.L. Brodsky. 2010. The endoplasmic reticulum-associated degradation of the epithelial sodium channel requires a unique complement of molecular chaperones. *Mol Biol Cell*. 21:1047-58.

Burda, P., S.M. Padilla, S. Sarkar, and S.D. Emr. 2002. Retromer function in endosome-to-Golgi retrograde transport is regulated by the yeast Vps34 PtdIns 3-kinase. *J Cell Sci*. 115:3889-900.

Canessa, C.M., A.M. Merillat, and B.C. Rossier. 1994a. Membrane topology of the epithelial sodium channel in intact cells. *Am J Physiol*. 267:C1682-90.

Canessa, C.M., L. Schild, G. Buell, B. Thorens, I. Gautschi, J.D. Horisberger, and B.C. Rossier. 1994b. Amiloride-sensitive epithelial Na+ channel is made of three homologous subunits. *Nature*. 367:463-7.

Caohuy, H., C. Jozwik, and H.B. Pollard. 2009. Rescue of DeltaF508-CFTR by the SGK1/Nedd4-2 signaling pathway. *J Biol Chem*. 284:25241-53.

Carattino, M.D., C.J. Passero, C.A. Steren, A.B. Maarouf, J.M. Pilewski, M.M. Myerburg, R.P. Hughey, and T.R. Kleyman. 2007. Defining an inhibitory domain in the alpha subunit of the epithelial sodium channel. *Am J Physiol Renal Physiol*.

Carlton, J.G., M. Agromayor, and J. Martin-Serrano. 2008. Differential requirements for Alix and ESCRT-III in cytokinesis and HIV-1 release. *Proc Natl Acad Sci U S A*. 105:10541-6.

Carlton, J.G., and J. Martin-Serrano. 2009. The ESCRT machinery: new functions in viral and cellular biology. *Biochem Soc Trans*. 37:195-9.

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

Carvalho, P., A.M. Stanley, and T.A. Rapoport. 2010. Retrotranslocation of a misfolded luminal ER protein by the ubiquitin-ligase Hrd1p. *Cell*. 143:579-91.

Casamayor, A., P.D. Torrance, T. Kobayashi, J. Thorner, and D.R. Alessi. 1999. Functional counterparts of mammalian protein kinases PDK1 and SGK in budding yeast. *Curr Biol*. 9:186-97.

Cazorla, M., M. Shegda, B. Ramesh, N.L. Harrison, and C. Kellendonk. 2012. Striatal D2 receptors regulate dendritic morphology of medium spiny neurons via Kir2 channels. *J Neurosci*. 32:2398-409.

Chang, A., and G.R. Fink. 1995. Targeting of the yeast plasma membrane [H+]ATPase: a novel gene AST1 prevents mislocalization of mutant ATPase to the vacuole. *J Cell Biol*. 128:39-49.

Chen, B., M. Retzlaff, T. Roos, and J. Frydman. 2011. Cellular strategies of protein quality control. *Cold Spring Harb Perspect Biol*. 3:a004374.

Chen, B.J., and R.A. Lamb. 2008. Mechanisms for enveloped virus budding: can some viruses do without an ESCRT? *Virology*. 372:221-32.

Cheng, S.H., R.J. Gregory, J. Marshall, S. Paul, D.W. Souza, G.A. White, C.R. O'Riordan, and A.E. Smith. 1990. Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell*. 63:827-34.

Chhangani, D., A.P. Joshi, and A. Mishra. 2012. E3 ubiquitin ligases in protein quality control mechanism. *Mol Neurobiol*. 45:571-85.

Chitra, S., G. Nalini, and G. Rajasekhar. 2012. The ubiquitin proteasome system and efficacy of proteasome inhibitors in diseases. *Int J Rheum Dis*. 15:249-60.

Choi, C., M. Davey, C. Schluter, P. Pandher, Y. Fang, L.J. Foster, and E. Conibear. 2011. Organization and assembly of the TRAPPII complex. *Traffic*. 12:715-25.

Chu, T., J. Sun, S. Saksena, and S.D. Emr. 2006. New component of ESCRT-I regulates endosomal sorting complex assembly. *J Cell Biol*. 175:815-23.

Claessen, J.H., L. Kundrat, and H.L. Ploegh. Protein quality control in the ER: balancing the ubiquitin checkbook. *Trends Cell Biol*. 22:22-32.

Claessen, J.H., L. Kundrat, and H.L. Ploegh. 2012. Protein quality control in the ER: balancing the ubiquitin checkbook. *Trends Cell Biol*. 22:22-32.

Colonna, T.E., L. Huynh, and D.M. Fambrough. 1997. Subunit interactions in the Na,K-ATPase explored with the yeast two-hybrid system. *J Biol Chem*. 272:12366-72.

Conibear, E., and T.H. Stevens. 2000. Vps52p, Vps53p, and Vps54p form a novel multisubunit complex required for protein sorting at the yeast late Golgi. *Mol Biol Cell*. 11:305-23.

Corbacho, I., I. Olivero, and L.M. Hernandez. 2005. A genome-wide screen for Saccharomyces cerevisiae nonessential genes involved in mannosyl phosphate transfer to mannoprotein-linked oligosaccharides. *Fungal Genet Biol*. 42:773-90.

Costanzo, M., A. Baryshnikova, J. Bellay, Y. Kim, E.D. Spear, C.S. Sevier, H. Ding, J.L. Koh, K. Toufighi, S. Mostafavi, J. Prinz, R.P. St Onge, B. VanderSluis, T. Makhnevych, F.J. Vizeacoumar, S. Alizadeh, S. Bahr, R.L. Brost, Y. Chen, M. Cokol, R. Deshpande, Z. Li, Z.Y. Lin, W. Liang, M. Marback, J. Paw, B.J. San Luis, E. Shuteriqi, A.H. Tong, N. van Dyk, I.M. Wallace, J.A. Whitney, M.T. Weirauch, G. Zhong, H. Zhu, W.A. Houry, M. Brudno, S. Ragibizadeh, B. Papp, C. Pal, F.P. Roth, G. Giaever, C. Nislow, O.G. Troyanskaya, H. Bussey, G.D. Bader, A.C. Gingras, Q.D. Morris, P.M. Kim, C.A. Kaiser, C.L. Myers, B.J. Andrews, and C. Boone. 2010. The genetic landscape of a cell. *Science*. 327:425-31.

Coughlan, C.M., J.L. Walker, J.C. Cochran, K.D. Wittrup, and J.L. Brodsky. 2004. Degradation of mutated bovine pancreatic trypsin inhibitor in the yeast vacuole suggests post-endoplasmic reticulum protein quality control. *J Biol Chem*. 279:15289-97.

Coury, L.A., J.C. Mathai, G.V. Prasad, J.L. Brodsky, P. Agre, and M.L. Zeidel. 1998. Reconstitution of water channel function of aquaporins 1 and 2 by expression in yeast secretory vesicles. *Am J Physiol*. 274:F34-42.

Csanady, L., P. Vergani, and D.C. Gadsby. 2010. Strict coupling between CFTR's catalytic cycle and gating of its Cl- ion pore revealed by distributions of open channel burst durations. *Proc Natl Acad Sci U S A*. 107:1241-6.

Curtiss, M., C. Jones, and M. Babst. 2007. Efficient cargo sorting by ESCRT-I and the subsequent release of ESCRT-I from multivesicular bodies requires the subunit Mvb12. *Mol Biol Cell*. 18:636-45.

Cyert, M.S., and C.C. Philpott. 2013. Regulation of Cation Balance in Saccharomyces cerevisiae. *Genetics*. 193:677-713.

D'Avanzo, N., W.W. Cheng, X. Xia, L. Dong, P. Savitsky, C.G. Nichols, and D.A. Doyle. 2010. Expression and purification of recombinant human inward rectifier K+ (KCNJ) channels in Saccharomyces cerevisiae. *Protein Expr Purif*. 71:115-21.

Dang, H., T.I. Klokk, B. Schaheen, B.M. McLaughlin, A.J. Thomas, T.A. Durns, B.G. Bitler, K. Sandvig, and H. Fares. 2011. Derlin-dependent retrograde transport from endosomes to the Golgi apparatus. *Traffic*. 12:1417-31.

Davis-Kaplan, S.R., M.A. Compton, A.R. Flannery, D.M. Ward, J. Kaplan, T.H. Stevens, and L.A. Graham. 2006. PKR1 encodes an assembly factor for the yeast V-type ATPase. *J Biol Chem*. 281:32025-35.

Davis, N.G., J.L. Horecka, and G.F. Sprague, Jr. 1993. Cis- and trans-acting functions required for endocytosis of the yeast pheromone receptors. *J Cell Biol*. 122:53-65.

de Boer, T.P., M.J. Houtman, M. Compier, and M.A. van der Heyden. 2010. The mammalian K(IR)2.x inward rectifier ion channel family: expression pattern and pathophysiology. *Acta Physiol (Oxf)*. 199:243-56.

DeCarvalho, A.C., L.J. Gansheroff, and J.L. Teem. 2002. Mutations in the nucleotide binding domain 1 signature motif region rescue processing and functional defects of cystic fibrosis transmembrane conductance regulator delta f508. *J Biol Chem*. 277:35896-905.

Denic, V., E.M. Quan, and J.S. Weissman. 2006. A luminal surveillance complex that selects misfolded glycoproteins for ER-associated degradation. *Cell*. 126:349-59.

Denis, C.L. 1984. Identification of new genes involved in the regulation of yeast alcohol dehydrogenase II. *Genetics*. 108:833-44.

Denning, G.M., M.P. Anderson, J.F. Amara, J. Marshall, A.E. Smith, and M.J. Welsh. 1992. Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. *Nature*. 358:761-4.

Deutschbauer, A.M., R.M. Williams, A.M. Chu, and R.W. Davis. 2002. Parallel phenotypic analysis of sporulation and postgermination growth in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*. 99:15530-5.

Dobson, C.M. 2003. Protein folding and misfolding. *Nature*. 426:884-90.

Dubouloz, F., O. Deloche, V. Wanke, E. Cameroni, and C. De Virgilio. 2005. The TOR and EGO protein complexes orchestrate microautophagy in yeast. *Mol Cell*. 19:15-26.

Dudek, J., J. Benedix, S. Cappel, M. Greiner, C. Jalal, L. Muller, and R. Zimmermann. 2009. Functions and pathologies of BiP and its interaction partners. *Cell Mol Life Sci*. 66:1556-69.

Dupre, S., and R. Haguenauer-Tsapis. 2001. Deubiquitination step in the endocytic pathway of yeast plasma membrane proteins: crucial role of Doa4p ubiquitin isopeptidase. *Mol Cell Biol*. 21:4482-94.

Dupre, S., D. Urban-Grimal, and R. Haguenauer-Tsapis. 2004. Ubiquitin and endocytic internalization in yeast and animal cells. *Biochim Biophys Acta*. 1695:89-111.

Dupressoir, A., A.P. Morel, W. Barbot, M.P. Loireau, L. Corbo, and T. Heidmann. 2001. Identification of four families of yCCR4- and Mg2+-dependent endonuclease-related proteins in higher eukaryotes, and characterization of orthologs of yCCR4 with a conserved leucine-rich repeat essential for hCAF1/hPOP2 binding. *BMC Genomics*. 2:9.

Eguez, L., Y.S. Chung, A. Kuchibhatla, M. Paidhungat, and S. Garrett. 2004. Yeast Mn2+ transporter, Smf1p, is regulated by ubiquitin-dependent vacuolar protein sorting. *Genetics*. 167:107-17.

Eitzen, G., E. Will, D. Gallwitz, A. Haas, and W. Wickner. 2000. Sequential action of two GTPases to promote vacuole docking and fusion. *EMBO J*. 19:6713-20.

Escola, J.M., M.J. Kleijmeer, W. Stoorvogel, J.M. Griffith, O. Yoshie, and H.J. Geuze. 1998. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J Biol Chem*. 273:20121-7.

Ferrari, P., M. Ferrandi, L. Torielli, P. Barassi, G. Tripodi, E. Minotti, I. Molinari, P. Melloni, and G. Bianchi. 2003. Antihypertensive compounds that modulate the Na-K pump. *Ann N Y Acad Sci*. 986:694-701.

Fiedler, B., and G. Scheiner-Bobis. 1996. Transmembrane topology of alpha- and beta-subunits of Na+,K+-ATPase derived from beta-galactosidase fusion proteins expressed in yeast. *J Biol Chem*. 271:29312-20.

Filimonenko, M., S. Stuffers, C. Raiborg, A. Yamamoto, L. Malerod, E.M. Fisher, A. Isaacs, A. Brech, H. Stenmark, and A. Simonsen. 2007. Functional multivesicular bodies are required for autophagic clearance of protein aggregates associated with neurodegenerative disease. *J Cell Biol*. 179:485-500.

Fink, M., F. Duprat, C. Heurteaux, F. Lesage, G. Romey, J. Barhanin, and M. Lazdunski. 1996. Dominant negative chimeras provide evidence for homo and heteromultimeric assembly of inward rectifier K+ channel proteins via their N-terminal end. *FEBS Lett*. 378:64-8.

Fisher, E.A., N.A. Khanna, and R.S. McLeod. 2011. Ubiquitination regulates the assembly of VLDL in HepG2 cells and is the committing step of the apoB-100 ERAD pathway. *J Lipid Res*. 52:1170-80.

Flegelova, H., R. Haguenauer-Tsapis, and H. Sychrova. 2006. Heterologous expression of mammalian Na/H antiporters in Saccharomyces cerevisiae. *Biochim Biophys Acta*. 1760:504-16.

Flegelova, H., and H. Sychrova. 2005. Mammalian NHE2 Na(+)/H+ exchanger mediates efflux of potassium upon heterologous expression in yeast. *FEBS Lett*. 579:4733-8.

Flynn, G.C., J. Pohl, M.T. Flocco, and J.E. Rothman. 1991. Peptide-binding specificity of the molecular chaperone BiP. *Nature*. 353:726-30.

Francis, G.S. 2008. The contemporary use of digoxin for the treatment of heart failure. *Circ Heart Fail*. 1:208-9.

Franssens, V., E. Boelen, J. Anandhakumar, T. Vanhelmont, S. Buttner, and J. Winderickx. 2009. Yeast unfolds the road map toward alpha-synuclein-induced cell death. *Cell Death Differ*. 17:746-53.

Fu, L., and E. Sztul. 2003. Traffic-independent function of the Sar1p/COPII machinery in proteasomal sorting of the cystic fibrosis transmembrane conductance regulator. *J Cell Biol*. 160:157-63.

Fu, L., and E. Sztul. 2009. ER-associated complexes (ERACs) containing aggregated cystic fibrosis transmembrane conductance regulator (CFTR) are degraded by autophagy. *Eur J Cell Biol*. 88:215-26.

Fujita, H., Y. Umezuki, K. Imamura, D. Ishikawa, S. Uchimura, A. Nara, T. Yoshimori, Y. Hayashizaki, J. Kawai, K. Ishidoh, Y. Tanaka, and M. Himeno. 2004. Mammalian class E Vps proteins, SBP1 and mVps2/CHMP2A, interact with and regulate the function of an AAA-ATPase SKD1/Vps4B. *J Cell Sci*. 117:2997-3009.

Gadsby, D.C. 2009. Ion channels versus ion pumps: the principal difference, in principle. *Nat Rev Mol Cell Biol*. 10:344-52.

Gao, M., and C.A. Kaiser. 2006. A conserved GTPase-containing complex is required for intracellular sorting of the general amino-acid permease in yeast. *Nat Cell Biol*. 8:657-67.

Garciadeblas, B., F. Rubio, F.J. Quintero, M.A. Banuelos, R. Haro, and A. Rodriguez-Navarro. 1993. Differential expression of two genes encoding isoforms of the ATPase involved in sodium efflux in Saccharomyces cerevisiae. *Mol Gen Genet*. 236:363-8.

Garrus, J.E., U.K. von Schwedler, O.W. Pornillos, S.G. Morham, K.H. Zavitz, H.E. Wang, D.A. Wettstein, K.M. Stray, M. Cote, R.L. Rich, D.G. Myszka, and W.I. Sundquist. 2001. Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. *Cell*. 107:55-65.

Geissler, S., K. Siegers, and E. Schiebel. 1998. A novel protein complex promoting formation of functional alpha- and gamma-tubulin. *EMBO J*. 17:952-66.

Gentzsch, M., H. Dang, Y. Dang, A. Garcia-Caballero, H. Suchindran, R.C. Boucher, and M.J. Stutts. 2010. The cystic fibrosis transmembrane conductance regulator impedes proteolytic stimulation of the epithelial Na+ channel. *J Biol Chem*. 285:32227-32.

Gestwicki, J.E., and D. Garza. 2012. Protein quality control in neurodegenerative disease. *Prog Mol Biol Transl Sci*. 107:327-53.

Ghaemmaghami, S., W.K. Huh, K. Bower, R.W. Howson, A. Belle, N. Dephoure, E.K. O'Shea, and J.S. Weissman. 2003. Global analysis of protein expression in yeast. *Nature*. 425:737-41.

Gill, D.J., H. Teo, J. Sun, O. Perisic, D.B. Veprintsev, S.D. Emr, and R.L. Williams. 2007. Structural insight into the ESCRT-I/-II link and its role in MVB trafficking. *EMBO J*. 26:600-12.

Giudicessi, J.R., and M.J. Ackerman. 2012. Potassium-channel mutations and cardiac arrhythmias--diagnosis and therapy. *Nat Rev Cardiol*. 9:319-32.

Gnann, A., J.R. Riordan, and D.H. Wolf. 2004. Cystic fibrosis transmembrane conductance regulator degradation depends on the lectins Htm1p/EDEM and the Cdc48 protein complex in yeast. *Mol Biol Cell*. 15:4125-35.

Goder, V. 2012. Roles of ubiquitin in endoplasmic reticulum-associated protein degradation (ERAD). *Curr Protein Pept Sci*. 13:425-35.

Goeckeler, J.L., and J.L. Brodsky. 2010. Molecular chaperones and substrate ubiquitination control the efficiency of endoplasmic reticulum-associated degradation. *Diabetes Obes Metab*. 12 Suppl 2:32-8.

Graves, F.M., and A. Tinker. 2000. Functional expression of the pore forming subunit of the ATP-sensitive potassium channel in Saccharomyces cerevisiae. *Biochem Biophys Res Commun*. 272:403-9.

Grishin, A., H. Li, E.S. Levitan, and E. Zaks-Makhina. 2006. Identification of gamma-aminobutyric acid receptor-interacting factor 1 (TRAK2) as a trafficking factor for the K+ channel Kir2.1. *J Biol Chem*. 281:30104-11.

Groll, M., L. Ditzel, J. Lowe, D. Stock, M. Bochtler, H.D. Bartunik, and R. Huber. 1997. Structure of 20S proteasome from yeast at 2.4 A resolution. *Nature*. 386:463-71.

Grove, D.E., M.F. Rosser, H.Y. Ren, A.P. Naren, and D.M. Cyr. 2009. Mechanisms for rescue of correctable folding defects in CFTRDelta F508. *Mol Biol Cell*. 20:4059-69.

Guerriero, C.J., and J.L. Brodsky. 2012. The delicate balance between secreted protein folding and endoplasmic reticulum-associated degradation in human physiology. *Physiol Rev*. 92:537-76.

Gui, Y.X., Y. Wan, Q. Xiao, Y. Wang, G. Wang, and S.D. Chen. 2011. Verification of expressions of Kir2 as potential peripheral biomarkers in lymphocytes from patients with Parkinson's disease. *Neurosci Lett*. 505:104-8.

Guizetti, J., L. Schermelleh, J. Mantler, S. Maar, I. Poser, H. Leonhardt, T. Muller-Reichert, and D.W. Gerlich. 2011. Cortical constriction during abscission involves helices of ESCRT-III-dependent filaments. *Science*. 331:1616-20.

Gupta, S.S., and C.M. Canessa. 2000. Heterologous expression of a mammalian epithelial sodium channel in yeast. *FEBS Lett*. 481:77-80.

Haass, F.A. 2007. Identification of yeast proteins necessary for cell surface function of a potassium channel. *In* Neuroscience. Vol. Doctor of Philosphy. University of California, San Francisco. 145.

Haass, F.A., M. Jonikas, P. Walter, J.S. Weissman, Y.N. Jan, L.Y. Jan, and M. Schuldiner. 2007. Identification of yeast proteins necessary for cell-surface function of a potassium channel. *Proc Natl Acad Sci U S A*. 104:18079-84.

Habeck, M., E. Cirri, A. Katz, S.J. Karlish, and H.J. Apell. 2009. Investigation of electrogenic partial reactions in detergent-solubilized Na,K-ATPase. *Biochemistry*. 48:9147-55.

Haft, C.R., M. de la Luz Sierra, R. Bafford, M.A. Lesniak, V.A. Barr, and S.I. Taylor. 2000. Human orthologs of yeast vacuolar protein sorting proteins Vps26, 29, and 35: assembly into multimeric complexes. *Mol Biol Cell*. 11:4105-16.

Hagiwara, S., and K. Takahashi. 1974. The anomalous rectification and cation selectivity of the membrane of a starfish egg cell. *J Membr Biol*. 18:61-80.

Halaban, R., E. Cheng, Y. Zhang, G. Moellmann, D. Hanlon, M. Michalak, V. Setaluri, and D.N. Hebert. 1997. Aberrant retention of tyrosinase in the endoplasmic reticulum mediates accelerated degradation of the enzyme and contributes to the dedifferentiated phenotype of amelanotic melanoma cells. *Proc Natl Acad Sci U S A*. 94:6210-5.

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

Han, S., Y. Liu, and A. Chang. 2007. Cytoplasmic Hsp70 promotes ubiquitination for endoplasmic reticulum-associated degradation of a misfolded mutant of the yeast plasma membrane ATPase, PMA1. *J Biol Chem*. 282:26140-9.

Hancock, L.C., R.P. Behta, and J.M. Lopes. 2006. Genomic analysis of the Opi- phenotype. *Genetics*. 173:621-34.

Hanwell, D., T. Ishikawa, R. Saleki, and D. Rotin. 2002. Trafficking and cell surface stability of the epithelial Na+ channel expressed in epithelial Madin-Darby canine kidney cells. *J Biol Chem*. 277:9772-9.

Haro, R., B. Garciadeblas, and A. Rodriguez-Navarro. 1991. A novel P-type ATPase from yeast involved in sodium transport. *FEBS Lett*. 291:189-91.

Hattori, T., T. Makiyama, M. Akao, E. Ehara, S. Ohno, M. Iguchi, Y. Nishio, K. Sasaki, H. Itoh, M. Yokode, T. Kita, M. Horie, and T. Kimura. 2012. A novel gain-of-function KCNJ2 mutation associated with short-QT syndrome impairs inward rectification of Kir2.1 currents. *Cardiovasc Res*. 93:666-73.

Hauck, C., T. Potter, M. Bartz, T. Wittwer, T. Wahlers, U. Mehlhorn, G. Scheiner-Bobis, A.A. McDonough, W. Bloch, R.H. Schwinger, and J. Muller-Ehmsen. 2009. Isoform specificity of cardiac glycosides binding to human Na+,K+-ATPase alpha1beta1, alpha2beta1 and alpha3beta1. *Eur J Pharmacol*. 622:7-14.

Hayes, M.J., K. Bryon, J. Satkurunathan, and T.P. Levine. 2011. Yeast homologues of three BLOC-1 subunits highlight KxDL proteins as conserved interactors of BLOC-1. *Traffic*. 12:260-8.

Haynes, C.M., S. Caldwell, and A.A. Cooper. 2002. An HRD/DER-independent ER quality control mechanism involves Rsp5p-dependent ubiquitination and ER-Golgi transport. *J Cell Biol*. 158:91-101.

Hebert, D.N., R. Bernasconi, and M. Molinari. 2010. ERAD substrates: which way out? *Semin Cell Dev Biol*. 21:526-32.

Hebert, D.N., and M. Molinari. 2012. Flagging and docking: dual roles for N-glycans in protein quality control and cellular proteostasis. *Trends Biochem Sci*. 37:404-10.

Heginbotham, L., Z. Lu, T. Abramson, and R. MacKinnon. 1994. Mutations in the K+ channel signature sequence. *Biophys J*. 66:1061-7.

Heinemeyer, W., M. Fischer, T. Krimmer, U. Stachon, and D.H. Wolf. 1997. The active sites of the eukaryotic 20 S proteasome and their involvement in subunit precursor processing. *J Biol Chem*. 272:25200-9.

Helliwell, S.B., S. Losko, and C.A. Kaiser. 2001. Components of a ubiquitin ligase complex specify polyubiquitination and intracellular trafficking of the general amino acid permease. *J Cell Biol*. 153:649-62.

Henne, W.M., N.J. Buchkovich, and S.D. Emr. 2011. The ESCRT pathway. *Dev Cell*. 21:77-91.

Henne, W.M., N.J. Buchkovich, Y. Zhao, and S.D. Emr. 2012. The endosomal sorting complex ESCRT-II mediates the assembly and architecture of ESCRT-III helices. *Cell*. 151:356-71.

Hettema, E.H., J. Valdez-Taubas, and H.R. Pelham. 2004. Bsd2 binds the ubiquitin ligase Rsp5 and mediates the ubiquitination of transmembrane proteins. *EMBO J*. 23:1279-88.

Hibino, H., A. Inanobe, K. Furutani, S. Murakami, I. Findlay, and Y. Kurachi. 2010. Inwardly rectifying potassium channels: their structure, function, and physiological roles. *Physiol Rev*. 90:291-366.

Hicke, L., and R. Dunn. 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu Rev Cell Dev Biol*. 19:141-72.

Hierro, A., J. Sun, A.S. Rusnak, J. Kim, G. Prag, S.D. Emr, and J.H. Hurley. 2004. Structure of the ESCRT-II endosomal trafficking complex. *Nature*. 431:221-5.

Hill, K., and A.A. Cooper. 2000. Degradation of unassembled Vph1p reveals novel aspects of the yeast ER quality control system. *EMBO J*. 19:550-61.

Holkeri, H., and M. Makarow. 1998. Different degradation pathways for heterologous glycoproteins in yeast. *FEBS Lett*. 429:162-6.

Hong, E., A.R. Davidson, and C.A. Kaiser. 1996. A pathway for targeting soluble misfolded proteins to the yeast vacuole. *J Cell Biol*. 135:623-33.

Horowitz, B., K.A. Eakle, G. Scheiner-Bobis, G.R. Randolph, C.Y. Chen, R.A. Hitzeman, and R.A. Farley. 1990. Synthesis and assembly of functional mammalian Na,K-ATPase in yeast. *J Biol Chem*. 265:4189-92.

Horowitz, B., and R.A. Farley. 1988. Development of a heterologous gene expression system for the Na,K-ATPase subunits in the yeast Saccharomyces cerevisiae. *Prog Clin Biol Res*. 268B:85-90.

Howard, T.L., D.R. Stauffer, C.R. Degnin, and S.M. Hollenberg. 2001. CHMP1 functions as a member of a newly defined family of vesicle trafficking proteins. *J Cell Sci*. 114:2395-404.

Howson, R., W.K. Huh, S. Ghaemmaghami, J.V. Falvo, K. Bower, A. Belle, N. Dephoure, D.D. Wykoff, J.S. Weissman, and E.K. O'Shea. 2005. Construction, verification and experimental use of two epitope-tagged collections of budding yeast strains. *Comp Funct Genomics*. 6:2-16.

Hu, B., X.L. Zhu, Q.X. Fan, H.X. Li, and C.W. Zou. 2012. Experimental study on inhibition of rat ventricular Ik1 by RNA interference targeting the KCNJ2 gene. *Biosci Trends*. 6:26-32.

Huang, C.F., L.M. Buu, W.L. Yu, and F.J. Lee. 1999. Characterization of a novel ADP-ribosylation factor-like protein (yARL3) in Saccharomyces cerevisiae. *J Biol Chem*. 274:3819-27.

Huang, C.L., S. Feng, and D.W. Hilgemann. 1998. Direct activation of inward rectifier potassium channels by PIP2 and its stabilization by Gbetagamma. *Nature*. 391:803-6.

Huber, R., B. Krueger, A. Diakov, J. Korbmacher, S. Haerteis, J. Einsiedel, P. Gmeiner, A.K. Azad, H. Cuppens, J.J. Cassiman, C. Korbmacher, and R. Rauh. 2010. Functional characterization of a partial loss-of-function mutation of the epithelial sodium channel (ENaC) associated with atypical cystic fibrosis. *Cell Physiol Biochem*. 25:145-58.

Hubner, C.A., and T.J. Jentsch. 2002. Ion channel diseases. *Hum Mol Genet*. 11:2435-45.

Hubner, C.A., and T.J. Jentsch. 2008. Channelopathies of transepithelial transport and vesicular function. *Adv Genet*. 63:113-52.

Hughey, R.P., G.M. Mueller, J.B. Bruns, C.L. Kinlough, P.A. Poland, K.L. Harkleroad, M.D. Carattino, and T.R. Kleyman. 2003. Maturation of the epithelial Na+ channel involves proteolytic processing of the alpha- and gamma-subunits. *J Biol Chem*. 278:37073-82.

Huh, W.K., J.V. Falvo, L.C. Gerke, A.S. Carroll, R.W. Howson, J.S. Weissman, and E.K. O'Shea. 2003. Global analysis of protein localization in budding yeast. *Nature*. 425:686-91.

Huotari, J., and A. Helenius. 2011. Endosome maturation. *EMBO J*. 30:3481-500.

Huyer, G., G.L. Longsworth, D.L. Mason, M.P. Mallampalli, J.M. McCaffery, R.L. Wright, and S. Michaelis. 2004a. A striking quality control subcompartment in Saccharomyces cerevisiae: the endoplasmic reticulum-associated compartment. *Mol Biol Cell*. 15:908-21.

Huyer, G., W.F. Piluek, Z. Fansler, S.G. Kreft, M. Hochstrasser, J.L. Brodsky, and S. Michaelis. 2004b. Distinct machinery is required in Saccharomyces cerevisiae for the endoplasmic

reticulum-associated degradation of a multispanning membrane protein and a soluble luminal protein. *J Biol Chem*. 279:38369-78.

Itakura, E., C. Kishi, K. Inoue, and N. Mizushima. 2008. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. *Mol Biol Cell*. 19:5360-72.

Jacobsen, M.D., P.A. Pedersen, and P.L. Jorgensen. 2002. Importance of Na,K-ATPase residue alpha 1-Arg544 in the segment Arg544-Asp567 for high-affinity binding of ATP, ADP, or MgATP. *Biochemistry*. 41:1451-6.

Jacquier, N., and R. Schneiter. 2010. Ypk1, the yeast orthologue of the human serum- and glucocorticoid-induced kinase, is required for efficient uptake of fatty acids. *J Cell Sci*. 123:2218-27.

Jansen, J.A., T.P. de Boer, R. Wolswinkel, T.A. van Veen, M.A. Vos, H.V. van Rijen, and M.A. van der Heyden. 2008. Lysosome mediated Kir2.1 breakdown directly influences inward rectifier current density. *Biochem Biophys Res Commun*. 367:687-92.

Jenness, D.D., Y. Li, C. Tipper, and P. Spatrick. 1997. Elimination of defective alpha-factor pheromone receptors. *Mol Cell Biol*. 17:6236-45.

Jensen, T.J., M.A. Loo, S. Pind, D.B. Williams, A.L. Goldberg, and J.R. Riordan. 1995. Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. *Cell*. 83:129-35.

Jin, H.S., K.W. Hong, J.E. Lim, S.Y. Hwang, S.H. Lee, C. Shin, H.K. Park, and B. Oh. 2010. Genetic variations in the sodium balance-regulating genes ENaC, NEDD4L, NDFIP2 and USP2 influence blood pressure and hypertension. *Kidney Blood Press Res*. 33:15-23.

Johnson, J.M., M. Jin, and D.J. Lew. 2011. Symmetry breaking and the establishment of cell polarity in budding yeast. *Curr Opin Genet Dev*. 21:740-6.

Jorgensen, J.R., and P.A. Pedersen. 2001. Role of phylogenetically conserved amino acids in folding of Na,K-ATPase. *Biochemistry*. 40:7301-8.

Jorgensen, P.L., J.R. Jorgensen, and P.A. Pedersen. 2001. Role of conserved TGDGVND-loop in Mg2+ binding, phosphorylation, and energy transfer in Na,K-ATPase. *J Bioenerg Biomembr*. 33:367-77.

Jorgensen, P.L., J.H. Rasmussen, J.M. Nielsen, and P.A. Pedersen. 1997. Transport-linked conformational changes in Na,K-ATPase. Structure-function relationships of ligand binding and E1-E2 conformational transitions. *Ann N Y Acad Sci*. 834:161-74.

Kabani, M., S.S. Kelley, M.W. Morrow, D.L. Montgomery, R. Sivendran, M.D. Rose, L.M. Gierasch, and J.L. Brodsky. 2003. Dependence of endoplasmic reticulum-associated degradation on the peptide binding domain and concentration of BiP. *Mol Biol Cell*. 14:3437-48.

Kabra, R., K.K. Knight, R. Zhou, and P.M. Snyder. 2008. Nedd4-2 induces endocytosis and degradation of proteolytically cleaved epithelial Na+ channels. *J Biol Chem*. 283:6033-9.

Kamura, T., D. Burian, H. Khalili, S.L. Schmidt, S. Sato, W.J. Liu, M.N. Conrad, R.C. Conaway, J.W. Conaway, and A. Shilatifard. 2001. Cloning and characterization of ELL-associated proteins EAP45 and EAP20. a role for yeast EAP-like proteins in regulation of gene expression by glucose. *J Biol Chem*. 276:16528-33.

Kaplan, J.H. 2002. Biochemistry of Na,K-ATPase. *Annu Rev Biochem*. 71:511-35.

Kashlan, O.B., G.M. Mueller, M.Z. Qamar, P.A. Poland, A. Ahner, R.C. Rubenstein, R.P. Hughey, J.L. Brodsky, and T.R. Kleyman. 2007. Small heat shock protein alphaA-crystallin regulates epithelial sodium channel expression. *J Biol Chem*. 282:28149-56.

Kato, M., and W. Wickner. 2003. Vam10p defines a Sec18p-independent step of priming that allows yeast vacuole tethering. *Proc Natl Acad Sci U S A*. 100:6398-403.

Katzmann, D.J., M. Babst, and S.D. Emr. 2001. Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. *Cell*. 106:145-55.

Katzmann, D.J., S. Sarkar, T. Chu, A. Audhya, and S.D. Emr. 2004. Multivesicular body sorting: ubiquitin ligase Rsp5 is required for the modification and sorting of carboxypeptidase S. *Mol Biol Cell*. 15:468-80.

Katzmann, D.J., C.J. Stefan, M. Babst, and S.D. Emr. 2003. Vps27 recruits ESCRT machinery to endosomes during MVB sorting. *J Cell Biol*. 162:413-23.

Kerr, M.C., J.S. Bennetts, F. Simpson, E.C. Thomas, C. Flegg, P.A. Gleeson, C. Wicking, and R.D. Teasdale. 2005. A novel mammalian retromer component, Vps26B. *Traffic*. 6:991-1001.

Kihara, A., T. Noda, N. Ishihara, and Y. Ohsumi. 2001. Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in Saccharomyces cerevisiae. *J Cell Biol*. 152:519-30.

Kim, Y., Y. Deng, and C.C. Philpott. 2007. GGA2- and ubiquitin-dependent trafficking of Arn1, the ferrichrome transporter of Saccharomyces cerevisiae. *Mol Biol Cell*. 18:1790-802.

Kincaid, M.M., and A.A. Cooper. 2007. Misfolded proteins traffic from the endoplasmic reticulum (ER) due to ER export signals. *Mol Biol Cell*. 18:455-63.

Kirchhausen, T., A.C. Davis, S. Frucht, B.O. Greco, G.S. Payne, and B. Tubb. 1991. AP17 and AP19, the mammalian small chains of the clathrin-associated protein complexes show homology to Yap17p, their putative homolog in yeast. *J Biol Chem*. 266:11153-7.

Kiser, G.L., M. Gentzsch, A.K. Kloser, E. Balzi, D.H. Wolf, A. Goffeau, and J.R. Riordan. 2001. Expression and degradation of the cystic fibrosis transmembrane conductance regulator in Saccharomyces cerevisiae. *Arch Biochem Biophys*. 390:195-205.

Klebl, F., and W. Tanner. 1989. Molecular cloning of a cell wall exo-beta-1,3-glucanase from Saccharomyces cerevisiae. *J Bacteriol*. 171:6259-64.

Kolacna, L., O. Zimmermannova, G. Hasenbrink, S. Schwarzer, J. Ludwig, H. Lichtenberg-Frate, and H. Sychrova. 2005. New phenotypes of functional expression of the mKir2.1 channel in potassium efflux-deficient Saccharomyces cerevisiae strains. *Yeast*. 22:1315-23.

Kolb, A.R., T.M. Buck, and J.L. Brodsky. 2011. Saccharomyces cerivisiae as a model system for kidney disease: what can yeast tell us about renal function? *Am J Physiol Renal Physiol*. 301:F1-11.

Kolehmainen, J., G.C. Black, A. Saarinen, K. Chandler, J. Clayton-Smith, A.L. Traskelin, R. Perveen, S. Kivitie-Kallio, R. Norio, M. Warburg, J.P. Fryns, A. de la Chapelle, and A.E. Lehesjoki. 2003. Cohen syndrome is caused by mutations in a novel gene, COH1, encoding a transmembrane protein with a presumed role in vesicle-mediated sorting and intracellular protein transport. *Am J Hum Genet*. 72:1359-69.

Konstas, A.A., C. Korbmacher, and S.J. Tucker. 2003. Identification of domains that control the heteromeric assembly of Kir5.1/Kir4.0 potassium channels. *Am J Physiol Cell Physiol*. 284:C910-7.

Korolchuk, V.I., M.M. Schutz, C. Gomez-Llorente, J. Rocha, N.R. Lansu, S.M. Collins, Y.P. Wairkar, I.M. Robinson, and C.J. O'Kane. 2007. Drosophila Vps35 function is necessary for normal endocytic trafficking and actin cytoskeleton organisation. *J Cell Sci*. 120:4367-76.

Kostelansky, M.S., C. Schluter, Y.Y. Tam, S. Lee, R. Ghirlando, B. Beach, E. Conibear, and J.H. Hurley. 2007. Molecular architecture and functional model of the complete yeast ESCRT-I heterotetramer. *Cell*. 129:485-98.

Kostova, Z., Y.C. Tsai, and A.M. Weissman. 2007. Ubiquitin ligases, critical mediators of endoplasmic reticulum-associated degradation. *Semin Cell Dev Biol*. 18:770-9.

Kraemer, C., T. Enklaar, B. Zabel, and E.R. Schmidt. 2000. Mapping and structure of DMXL1, a human homologue of the DmX gene from Drosophila melanogaster coding for a WD repeat protein. *Genomics*. 64:97-101.

Kruse, K.B., J.L. Brodsky, and A.A. McCracken. 2006. Characterization of an ERAD gene as VPS30/ATG6 reveals two alternative and functionally distinct protein quality control pathways: one for soluble Z variant of human alpha-1 proteinase inhibitor (A1PiZ) and another for aggregates of A1PiZ. *Mol Biol Cell*. 17:203-12.

Kubo, Y., T.J. Baldwin, Y.N. Jan, and L.Y. Jan. 1993. Primary structure and functional expression of a mouse inward rectifier potassium channel. *Nature*. 362:127-33.

Kuwahara, M., I. Shinbo, K. Sato, Y. Terada, F. Marumo, and S. Sasaki. 1999. Transmembrane helix 5 is critical for the high water permeability of aquaporin. *Biochemistry*. 38:16340-6.

Laize, V., P. Ripoche, and F. Tacnet. 1997. Purification and functional reconstitution of the human CHIP28 water channel expressed in Saccharomyces cerevisiae. *Protein Expr Purif*. 11:284-8.

Laize, V., G. Rousselet, J.M. Verbavatz, V. Berthonaud, R. Gobin, N. Roudier, L. Abrami, P. Ripoche, and F. Tacnet. 1995. Functional expression of the human CHIP28 water channel in a yeast secretory mutant. *FEBS Lett*. 373:269-74.

Lang, F., F. Artunc, and V. Vallon. 2009. The physiological impact of the serum and glucocorticoid-inducible kinase SGK1. *Curr Opin Nephrol Hypertens*. 18:439-48.

Laufen, T., M.P. Mayer, C. Beisel, D. Klostermeier, A. Mogk, J. Reinstein, and B. Bukau. 1999. Mechanism of regulation of hsp70 chaperones by DnaJ cochaperones. *Proc Natl Acad Sci U S A*. 96:5452-7.

Lee, F.J., C.F. Huang, W.L. Yu, L.M. Buu, C.Y. Lin, M.C. Huang, J. Moss, and M. Vaughan. 1997. Characterization of an ADP-ribosylation factor-like 1 protein in Saccharomyces cerevisiae. *J Biol Chem*. 272:30998-1005.

Lee, M.W., B.J. Kim, H.K. Choi, M.J. Ryu, S.B. Kim, K.M. Kang, E.J. Cho, H.D. Youn, W.K. Huh, and S.T. Kim. 2007. Global protein expression profiling of budding yeast in response to DNA damage. *Yeast*. 24:145-54.

Lehle, L., S. Strahl, and W. Tanner. 2006. Protein glycosylation, conserved from yeast to man: a model organism helps elucidate congenital human diseases. *Angew Chem Int Ed Engl*. 45:6802-18.

Lenk, U., H. Yu, J. Walter, M.S. Gelman, E. Hartmann, R.R. Kopito, and T. Sommer. 2002. A role for mammalian Ubc6 homologues in ER-associated protein degradation. *J Cell Sci*. 115:3007-14.

Leonoudakis, D., L.R. Conti, S. Anderson, C.M. Radeke, L.M. McGuire, M.E. Adams, S.C. Froehner, J.R. Yates, 3rd, and C.A. Vandenberg. 2004. Protein trafficking and anchoring complexes revealed by proteomic analysis of inward rectifier potassium channel (Kir2.x)-associated proteins. *J Biol Chem*. 279:22331-46.

Levey, A.S., L.A. Stevens, C.H. Schmid, Y.L. Zhang, A.F. Castro, 3rd, H.I. Feldman, J.W. Kusek, P. Eggers, F. Van Lente, T. Greene, and J. Coresh. 2009. A new equation to estimate glomerular filtration rate. *Ann Intern Med*. 150:604-12.

Leyland, M.L., and C. Dart. 2004. An alternatively spliced isoform of PSD-93/chapsyn 110 binds to the inwardly rectifying potassium channel, Kir2.1. *J Biol Chem*. 279:43427-36.

Li, B., and J.R. Warner. 1996. Mutation of the Rab6 homologue of Saccharomyces cerevisiae, YPT6, inhibits both early Golgi function and ribosome biosynthesis. *J Biol Chem*. 271:16813-9.

Li, L., and S.N. Cohen. 1996. Tsg101: a novel tumor susceptibility gene isolated by controlled homozygous functional knockout of allelic loci in mammalian cells. *Cell*. 85:319-29.

Li, Y., T. Kane, C. Tipper, P. Spatrick, and D.D. Jenness. 1999. Yeast mutants affecting possible quality control of plasma membrane proteins. *Mol Cell Biol*. 19:3588-99.

Li, Z., S. Bahr, E. Shuteriqi, and C. Boone. 2010. (2010). Boone Lab Research Projects - Genetic Networks: Temperature Sensitive Conditional Mutant Collection [online]. University of Toronto. http://www.utoronto.ca/boonelab/research_projects/genetic_networks/temp_sensitive_mutants/index.shtml [2011].

Li, Z., F.J. Vizeacoumar, S. Bahr, J. Li, J. Warringer, F.S. Vizeacoumar, R. Min, B. Vandersluis, J. Bellay, M. Devit, J.A. Fleming, A. Stephens, J. Haase, Z.Y. Lin, A. Baryshnikova, H. Lu, Z. Yan, K. Jin, S. Barker, A. Datti, G. Giaever, C. Nislow, C. Bulawa, C.L. Myers, M. Costanzo, A.C. Gingras, Z. Zhang, A. Blomberg, K. Bloom, B. Andrews, and C. Boone. 2011. Systematic exploration of essential yeast gene function with temperature-sensitive mutants. *Nat Biotechnol*. 29:361-7.

Liang, H., C.H. Ko, T. Herman, and R.F. Gaber. 1998. Trinucleotide insertions, deletions, and point mutations in glucose transporters confer K+ uptake in Saccharomyces cerevisiae. *Mol Cell Biol*. 18:926-35.

Liewen, H., I. Meinhold-Heerlein, V. Oliveira, R. Schwarzenbacher, G. Luo, A. Wadle, M. Jung, M. Pfreundschuh, and F. Stenner-Liewen. 2005. Characterization of the human GARP (Golgi associated retrograde protein) complex. *Exp Cell Res*. 306:24-34.

Lingrel, J.B. 2010. The physiological significance of the cardiotonic steroid/ouabain-binding site of the Na,K-ATPase. *Annu Rev Physiol*. 72:395-412.

Lingrel, J.B., J. Van Huysse, W. O'Brien, E. Jewell-Motz, and P. Schultheis. 1994. Na,K-ATPase: structure-function studies. *Ren Physiol Biochem*. 17:198-200.

Liu, X.F., F. Supek, N. Nelson, and V.C. Culotta. 1997. Negative control of heavy metal uptake by the Saccharomyces cerevisiae BSD2 gene. *J Biol Chem*. 272:11763-9.

Liu, Z., J. Shen, J.M. Carbrey, R. Mukhopadhyay, P. Agre, and B.P. Rosen. 2002. Arsenite transport by mammalian aquaglyceroporins AQP7 and AQP9. *Proc Natl Acad Sci U S A*. 99:6053-8.

Loffing, J., S.Y. Flores, and O. Staub. 2006. Sgk kinases and their role in epithelial transport. *Annu Rev Physiol*. 68:461-90.

Loh, E., and W. Hong. 2004. The binary interacting network of the conserved oligomeric Golgi tethering complex. *J Biol Chem*. 279:24640-8.

Loo, M.A., T.J. Jensen, L. Cui, Y. Hou, X.B. Chang, and J.R. Riordan. 1998. Perturbation of Hsp90 interaction with nascent CFTR prevents its maturation and accelerates its degradation by the proteasome. *EMBO J*. 17:6879-87.

Lopatin, A.N., E.N. Makhina, and C.G. Nichols. 1994. Potassium channel block by cytoplasmic polyamines as the mechanism of intrinsic rectification. *Nature*. 372:366-9.

Lopes, C.M., H. Zhang, T. Rohacs, T. Jin, J. Yang, and D.E. Logothetis. 2002. Alterations in conserved Kir channel-PIP2 interactions underlie channelopathies. *Neuron*. 34:933-44.

Lourdel, S., M. Paulais, F. Cluzeaud, M. Bens, M. Tanemoto, Y. Kurachi, A. Vandewalle, and J. Teulon. 2002. An inward rectifier K(+) channel at the basolateral membrane of the mouse distal convoluted tubule: similarities with Kir4-Kir5.1 heteromeric channels. *J Physiol*. 538:391-404.

Lowe, J., D. Stock, B. Jap, P. Zwickl, W. Baumeister, and R. Huber. 1995. Crystal structure of the 20S proteasome from the archaeon T. acidophilum at 3.4 A resolution. *Science*. 268:533-9.

Lu, C., C. Jiang, S. Pribanic, and D. Rotin. 2007. CFTR stabilizes ENaC at the plasma membrane. *J Cyst Fibros*. 6:419-22.

Luciani, A., V.R. Villella, S. Esposito, N. Brunetti-Pierri, D. Medina, C. Settembre, M. Gavina, L. Pulze, I. Giardino, M. Pettoello-Mantovani, M. D'Apolito, S. Guido, E. Masliah, B. Spencer, S. Quaratino, V. Raia, A. Ballabio, and L. Maiuri. 2010. Defective CFTR induces aggresome formation and lung inflammation in cystic fibrosis through ROS-mediated autophagy inhibition. *Nat Cell Biol*. 12:863-75.

Luhtala, N., and G. Odorizzi. 2004. Bro1 coordinates deubiquitination in the multivesicular body pathway by recruiting Doa4 to endosomes. *J Cell Biol*. 166:717-29.

Luke, M.M., F. Della Seta, C.J. Di Como, H. Sugimoto, R. Kobayashi, and K.T. Arndt. 1996. The SAP, a new family of proteins, associate and function positively with the SIT4 phosphatase. *Mol Cell Biol*. 16:2744-55.

Luo, W., and A. Chang. 1997. Novel genes involved in endosomal traffic in yeast revealed by suppression of a targeting-defective plasma membrane ATPase mutant. *J Cell Biol*. 138:731-46.

Luo, Z., and D. Gallwitz. 2003. Biochemical and genetic evidence for the involvement of yeast Ypt6-GTPase in protein retrieval to different Golgi compartments. *J Biol Chem*. 278:791-9.

Lussier, M., A.M. White, J. Sheraton, T. di Paolo, J. Treadwell, S.B. Southard, C.I. Horenstein, J. Chen-Weiner, A.F. Ram, J.C. Kapteyn, T.W. Roemer, D.H. Vo, D.C. Bondoc, J. Hall, W.W. Zhong, A.M. Sdicu, J. Davies, F.M. Klis, P.W. Robbins, and H. Bussey. 1997. Large scale identification of genes involved in cell surface biosynthesis and architecture in Saccharomyces cerevisiae. *Genetics*. 147:435-50.

Ma, D., T.K. Taneja, B.M. Hagen, B.Y. Kim, B. Ortega, W.J. Lederer, and P.A. Welling. 2011. Golgi export of the Kir2.1 channel is driven by a trafficking signal located within its tertiary structure. *Cell*. 145:1102-15.

Ma, D., N. Zerangue, Y.F. Lin, A. Collins, M. Yu, Y.N. Jan, and L.Y. Jan. 2001. Role of ER export signals in controlling surface potassium channel numbers. *Science*. 291:316-9.

MacGurn, J.A., P.C. Hsu, and S.D. Emr. 2012. Ubiquitin and membrane protein turnover: from cradle to grave. *Annu Rev Biochem*. 81:231-59.

Makuc, J., C. Cappellaro, and E. Boles. 2004. Co-expression of a mammalian accessory trafficking protein enables functional expression of the rat MCT1 monocarboxylate transporter in Saccharomyces cerevisiae. *FEMS Yeast Res*. 4:795-801.

Malik, B., S.R. Price, W.E. Mitch, Q. Yue, and D.C. Eaton. 2006. Regulation of epithelial sodium channels by the ubiquitin-proteasome proteolytic pathway. *Am J Physiol Renal Physiol*. 290:F1285-94.

Malik, B., L. Schlanger, O. Al-Khalili, H.F. Bao, G. Yue, S.R. Price, W.E. Mitch, and D.C. Eaton. 2001. Enac degradation in A6 cells by the ubiquitin-proteosome proteolytic pathway. *J Biol Chem*. 276:12903-10.

Malo, M.E., and L. Fliegel. 2006. Physiological role and regulation of the Na+/H+ exchanger. *Can J Physiol Pharmacol*. 84:1081-95.

Marini, A.M., G. Matassi, V. Raynal, B. Andre, J.P. Cartron, and B. Cherif-Zahar. 2000. The human Rhesus-associated RhAG protein and a kidney homologue promote ammonium transport in yeast. *Nat Genet*. 26:341-4.

Martin, A., T.A. Baker, and R.T. Sauer. 2008. Pore loops of the AAA+ ClpX machine grip substrates to drive translocation and unfolding. *Nat Struct Mol Biol*. 15:1147-51.

Mathai, J.C., and P. Agre. 1999. Hourglass pore-forming domains restrict aquaporin-1 tetramer assembly. *Biochemistry*. 38:923-8.

Matsuda, H., A. Saigusa, and H. Irisawa. 1987. Ohmic conductance through the inwardly rectifying K channel and blocking by internal Mg2+. *Nature*. 325:156-9.

Matyskiela, M.E., and A. Martin. 2013. Design principles of a universal protein degradation machine. *J Mol Biol*. 425:199-213.

May, C.W., and M.N. Diaz. 2008. The role of digoxin in the treatment of heart failure. *Circ Heart Fail*. 1:206-7.

Mayer, M.P., and B. Bukau. 2005. Hsp70 chaperones: cellular functions and molecular mechanism. *Cell Mol Life Sci*. 62:670-84.

Mayers, J.R., I. Fyfe, A.L. Schuh, E.R. Chapman, J.M. Edwardson, and A. Audhya. 2011. ESCRT-0 assembles as a heterotetrameric complex on membranes and binds multiple ubiquitinylated cargoes simultaneously. *J Biol Chem*. 286:9636-45.

McCarty, J.S., A. Buchberger, J. Reinstein, and B. Bukau. 1995. The role of ATP in the functional cycle of the DnaK chaperone system. *J Mol Biol*. 249:126-37.

McCracken, A.A., and J.L. Brodsky. 1996. Assembly of ER-associated protein degradation in vitro: dependence on cytosol, calnexin, and ATP. *J Cell Biol*. 132:291-8.

McCullough, J., P.E. Row, O. Lorenzo, M. Doherty, R. Beynon, M.J. Clague, and S. Urbe. 2006. Activation of the endosome-associated ubiquitin isopeptidase AMSH by STAM, a component of the multivesicular body-sorting machinery. *Curr Biol*. 16:160-5.

McLerie, M., and A.N. Lopatin. 2003. Dominant-negative suppression of I(K1) in the mouse heart leads to altered cardiac excitability. *J Mol Cell Cardiol*. 35:367-78.

McNew, J.A., J.G. Coe, M. Sogaard, B.V. Zemelman, C. Wimmer, W. Hong, and T.H. Sollner. 1998. Gos1p, a Saccharomyces cerevisiae SNARE protein involved in Golgi transport. *FEBS Lett*. 435:89-95.

McNew, J.A., M. Sogaard, N.M. Lampen, S. Machida, R.R. Ye, L. Lacomis, P. Tempst, J.E. Rothman, and T.H. Sollner. 1997. Ykt6p, a prenylated SNARE essential for endoplasmic reticulum-Golgi transport. *J Biol Chem*. 272:17776-83.

Mehnert, M., T. Sommer, and E. Jarosch. 2010. ERAD ubiquitin ligases: multifunctional tools for protein quality control and waste disposal in the endoplasmic reticulum. *Bioessays*. 32:905-13.

Meiling-Wesse, K., U.D. Epple, R. Krick, H. Barth, A. Appelles, C. Voss, E.L. Eskelinen, and M. Thumm. 2005. Trs85 (Gsg1), a component of the TRAPP complexes, is required for the organization of the preautophagosomal structure during selective autophagy via the Cvt pathway. *J Biol Chem*. 280:33669-78.

Meyer, H.H., Y. Wang, and G. Warren. 2002. Direct binding of ubiquitin conjugates by the mammalian p97 adaptor complexes, p47 and Ufd1-Npl4. *EMBO J*. 21:5645-52.

Miller, V.J., and D. Ungar. 2012. Re'COG'nition at the Golgi. *Traffic*. 13:891-7.

Minor, D.L., Jr., S.J. Masseling, Y.N. Jan, and L.Y. Jan. 1999. Transmembrane structure of an inwardly rectifying potassium channel. *Cell*. 96:879-91.

Mitchell, A.G., and C.E. Martin. 1997. Fah1p, a Saccharomyces cerevisiae cytochrome b5 fusion protein, and its Arabidopsis thaliana homolog that lacks the cytochrome b5 domain both function in the alpha-hydroxylation of sphingolipid-associated very long chain fatty acids. *J Biol Chem*. 272:28281-8.

Mitchell, D.M., M. Zhou, R. Pariyarath, H. Wang, J.D. Aitchison, H.N. Ginsberg, and E.A. Fisher. 1998. Apoprotein B100 has a prolonged interaction with the translocon during

which its lipidation and translocation change from dependence on the microsomal triglyceride transfer protein to independence. *Proc Natl Acad Sci U S A*. 95:14733-8.

Mizushima, N. 2011. Autophagy in protein and organelle turnover. *Cold Spring Harb Symp Quant Biol*. 76:397-402.

Montero-Lomeli, M., and A.L. Okorokova Facanha. 1999. Expression of a mammalian Na+/H+ antiporter in Saccharomyces cerevisiae. *Biochem Cell Biol*. 77:25-31.

Montpetit, B., and E. Conibear. 2009. Identification of the novel TRAPP associated protein Tca17. *Traffic*. 10:713-23.

Mouyna, I., R.P. Hartland, T. Fontaine, M. Diaquin, C. Simenel, M. Delepierre, B. Henrissat, and J.P. Latge. 1998. A 1,3-beta-glucanosyltransferase isolated from the cell wall of Aspergillus fumigatus is a homologue of the yeast Bgl2p. *Microbiology*. 144 (Pt 11):3171-80.

Mrsa, V., F. Klebl, and W. Tanner. 1993. Purification and characterization of the Saccharomyces cerevisiae BGL2 gene product, a cell wall endo-beta-1,3-glucanase. *J Bacteriol*. 175:2102-6.

Mueller, G.M., A.B. Maarouf, C.L. Kinlough, N. Sheng, O.B. Kashlan, S. Okumura, S. Luthy, T.R. Kleyman, and R.P. Hughey. 2010. Cys palmitoylation of the beta subunit modulates gating of the epithelial sodium channel. *J Biol Chem*. 285:30453-62.

Muller-Ehmsen, J., P. Juvvadi, C.B. Thompson, L. Tumyan, M. Croyle, J.B. Lingrel, R.H. Schwinger, A.A. McDonough, and R.A. Farley. 2001. Ouabain and substrate affinities of human Na(+)-K(+)-ATPase alpha(1)beta(1), alpha(2)beta(1), and alpha(3)beta(1) when expressed separately in yeast cells. *Am J Physiol Cell Physiol*. 281:C1355-64.

Mumberg, D., R. Muller, and M. Funk. 1994. Regulatable promoters of Saccharomyces cerevisiae: comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Res*. 22:5767-8.

Mumberg, D., R. Muller, and M. Funk. 1995. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene*. 156:119-22.

Munson, A.M., D.H. Haydon, S.L. Love, G.L. Fell, V.R. Palanivel, and A.G. Rosenwald. 2004. Yeast ARL1 encodes a regulator of K+ influx. *J Cell Sci*. 117:2309-20.

Nakamura, R.L., and R.F. Gaber. 1998. Studying ion channels using yeast genetics. *Methods Enzymol*. 293:89-104.

Nakashima, N., E. Noguchi, and T. Nishimoto. 1999. Saccharomyces cerevisiae putative G protein, Gtr1p, which forms complexes with itself and a novel protein designated as Gtr2p, negatively regulates the Ran/Gsp1p G protein cycle through Gtr2p. *Genetics*. 152:853-67.

Nakatsukasa, K., G. Huyer, S. Michaelis, and J.L. Brodsky. 2008. Dissecting the ER-associated degradation of a misfolded polytopic membrane protein. *Cell*. 132:101-12.

Nalos, L., T.P. de Boer, M.J. Houtman, M.B. Rook, M.A. Vos, and M.A. van der Heyden. 2011. Inhibition of lysosomal degradation rescues pentamidine-mediated decreases of K(IR)2.1 ion channel expression but not that of K(v)11.1. *Eur J Pharmacol*. 652:96-103.

Needham, P.G., K. Mikoluk, P. Dhakarwal, S. Khadem, A.C. Snyder, A.R. Subramanya, and J.L. Brodsky. 2011. The thiazide-sensitive NaCl cotransporter is targeted for chaperone-dependent endoplasmic reticulum-associated degradation. *J Biol Chem*. 286:43611-21.

Nehring, R.B., E. Wischmeyer, F. Doring, R.W. Veh, M. Sheng, and A. Karschin. 2000. Neuronal inwardly rectifying K(+) channels differentially couple to PDZ proteins of the PSD-95/SAP90 family. *J Neurosci*. 20:156-62.

Ng, K.E., S. Schwarzer, M.R. Duchen, and A. Tinker. 2010. The intracellular localization and function of the ATP-sensitive K+ channel subunit Kir6.1. *J Membr Biol*. 234:137-47.

Nickell, S., F. Beck, S.H. Scheres, A. Korinek, F. Forster, K. Lasker, O. Mihalache, N. Sun, I. Nagy, A. Sali, J.M. Plitzko, J.M. Carazo, M. Mann, and W. Baumeister. 2009. Insights into the molecular architecture of the 26S proteasome. *Proc Natl Acad Sci U S A*. 106:11943-7.

Nickerson, D.P., M. West, and G. Odorizzi. 2006. Did2 coordinates Vps4-mediated dissociation of ESCRT-III from endosomes. *J Cell Biol*. 175:715-20.

Nielsen, J.M., P.A. Pedersen, S.J. Karlish, and P.L. Jorgensen. 1998. Importance of intramembrane carboxylic acids for occlusion of K+ ions at equilibrium in renal Na,K-ATPase. *Biochemistry*. 37:1961-8.

Nishikawa, S.I., S.W. Fewell, Y. Kato, J.L. Brodsky, and T. Endo. 2001. Molecular chaperones in the yeast endoplasmic reticulum maintain the solubility of proteins for retrotranslocation and degradation. *J Cell Biol*. 153:1061-70.

Noble, D. 1965. Electrical properties of cardiac muscle attributable to inward going (anamalous) rectification. *Journal of Cellular and Comparative Physiology*. 66:127-135.

Noda, Y., E. Sohara, E. Ohta, and S. Sasaki. 2010. Aquaporins in kidney pathophysiology. *Nat Rev Nephrol*. 6:168-78.

Nothwehr, S.F., N.J. Bryant, and T.H. Stevens. 1996. The newly identified yeast GRD genes are required for retention of late-Golgi membrane proteins. *Mol Cell Biol*. 16:2700-7.

Nothwehr, S.F., S.A. Ha, and P. Bruinsma. 2000. Sorting of yeast membrane proteins into an endosome-to-Golgi pathway involves direct interaction of their cytosolic domains with Vps35p. *J Cell Biol*. 151:297-310.

Obara, K., T. Sekito, and Y. Ohsumi. 2006. Assortment of phosphatidylinositol 3-kinase complexes--Atg14p directs association of complex I to the pre-autophagosomal structure in Saccharomyces cerevisiae. *Mol Biol Cell*. 17:1527-39.

Okiyoneda, T., H. Barriere, M. Bagdany, W.M. Rabeh, K. Du, J. Hohfeld, J.C. Young, and G.L. Lukacs. 2010. Peripheral protein quality control removes unfolded CFTR from the plasma membrane. *Science*. 329:805-10.

Ouyang, Y., Q. Xu, K. Mitsui, M. Motizuki, and Z. Xu. 2009. Human trehalase is a stress responsive protein in Saccharomyces cerevisiae. *Biochem Biophys Res Commun*. 379:621-5.

Paddon, C., D. Loayza, L. Vangelista, R. Solari, and S. Michaelis. 1996. Analysis of the localization of STE6/CFTR chimeras in a Saccharomyces cerevisiae model for the cystic fibrosis defect CFTR delta F508. *Mol Microbiol*. 19:1007-17.

Pagant, S., L. Kung, M. Dorrington, M.C. Lee, and E.A. Miller. 2007. Inhibiting endoplasmic reticulum (ER)-associated degradation of misfolded Yor1p does not permit ER export despite the presence of a diacidic sorting signal. *Mol Biol Cell*. 18:3398-413.

Pan, X., P. Roberts, Y. Chen, E. Kvam, N. Shulga, K. Huang, S. Lemmon, and D.S. Goldfarb. 2000. Nucleus-vacuole junctions in Saccharomyces cerevisiae are formed through the direct interaction of Vac8p with Nvj1p. *Mol Biol Cell*. 11:2445-57.

Park, J.S., and A.M. Neiman. 2012. VPS13 regulates membrane morphogenesis during sporulation in Saccharomyces cerevisiae. *J Cell Sci*. 125:3004-11.

Paynter, J.J., L. Shang, M.K. Bollepalli, T. Baukrowitz, and S.J. Tucker. 2010. Random mutagenesis screening indicates the absence of a separate H(+)-sensor in the pH-sensitive Kir channels. *Channels (Austin)*. 4:390-7.

Pedersen, A., and P.L. Jorgensen. 1992. Expression of Na,K-ATPase in Saccharomyces cerevisiae. *Ann N Y Acad Sci*. 671:452-4.

Pedersen, P.A., J.R. Jorgensen, and P.L. Jorgensen. 2000. Importance of conserved alpha - subunit segment 709GDGVND for Mg2+ binding, phosphorylation, and energy transduction in Na,K-ATPase. *J Biol Chem*. 275:37588-95.

Pedersen, P.A., J.M. Nielsen, J.H. Rasmussen, and P.L. Jorgensen. 1998. Contribution to Tl+, K+, and Na+ binding of Asn776, Ser775, Thr774, Thr772, and Tyr771 in cytoplasmic part of fifth transmembrane segment in alpha-subunit of renal Na,K-ATPase. *Biochemistry*. 37:17818-27.

Pedersen, P.A., J.H. Rasmussen, and P.L. Joorgensen. 1996a. Expression in high yield of pig alpha 1 beta 1 Na,K-ATPase and inactive mutants D369N and D807N in Saccharomyces cerevisiae. *J Biol Chem*. 271:2514-22.

Pedersen, P.A., J.H. Rasmussen, and P.L. Jorgensen. 1996b. Consequences of mutations to the phosphorylation site of the alpha-subunit of Na, K-ATPase for ATP binding and E1-E2 conformational equilibrium. *Biochemistry*. 35:16085-93.

Pedersen, P.A., J.H. Rasmussen, and P.L. Jorgensen. 1997a. Increase in affinity for ATP and change in E1-E2 conformational equilibrium after mutations to the phosphorylation site (Asp369) of the alpha subunit of Na,K-ATPase. *Ann N Y Acad Sci*. 834:454-6.

Pedersen, P.A., J.H. Rasmussen, J.M. Nielsen, and P.L. Jorgensen. 1997b. Identification of Asp804 and Asp808 as Na+ and K+ coordinating residues in alpha-subunit of renal Na,K-ATPase. *FEBS Lett*. 400:206-10.

Pettersson, N., J. Hagstrom, R.M. Bill, and S. Hohmann. 2006. Expression of heterologous aquaporins for functional analysis in Saccharomyces cerevisiae. *Curr Genet*. 50:247-55.

Phan, H.L., J.A. Finlay, D.S. Chu, P.K. Tan, T. Kirchhausen, and G.S. Payne. 1994. The Saccharomyces cerevisiae APS1 gene encodes a homolog of the small subunit of the mammalian clathrin AP-1 complex: evidence for functional interaction with clathrin at the Golgi complex. *EMBO J*. 13:1706-17.

Pierri, J.N., C.L. Volk, S. Auh, A. Sampson, and D.A. Lewis. 2003. Somal size of prefrontal cortical pyramidal neurons in schizophrenia: differential effects across neuronal subpopulations. *Biol Psychiatry*. 54:111-20.

Pilewski, J.M., and R.A. Frizzell. 1999. Role of CFTR in airway disease. *Physiol Rev*. 79:S215-55.

Pizzirusso, M., and A. Chang. 2004. Ubiquitin-mediated targeting of a mutant plasma membrane ATPase, Pma1-7, to the endosomal/vacuolar system in yeast. *Mol Biol Cell*. 15:2401-9.

Plaster, N.M., R. Tawil, M. Tristani-Firouzi, S. Canun, S. Bendahhou, A. Tsunoda, M.R. Donaldson, S.T. Iannaccone, E. Brunt, R. Barohn, J. Clark, F. Deymeer, A.L. George, Jr., F.A. Fish, A. Hahn, A. Nitu, C. Ozdemir, P. Serdaroglu, S.H. Subramony, G. Wolfe, Y.H. Fu, and L.J. Ptacek. 2001. Mutations in Kir2.1 cause the developmental and episodic electrical phenotypes of Andersen's syndrome. *Cell*. 105:511-9.

Plemper, R.K., S. Bohmler, J. Bordallo, T. Sommer, and D.H. Wolf. 1997. Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. *Nature*. 388:891-5.

Plemper, R.K., R. Egner, K. Kuchler, and D.H. Wolf. 1998. Endoplasmic reticulum degradation of a mutated ATP-binding cassette transporter Pdr5 proceeds in a concerted action of Sec61 and the proteasome. *J Biol Chem*. 273:32848-56.

Polevoda, B., and F. Sherman. 2001. NatC Nalpha-terminal acetyltransferase of yeast contains three subunits, Mak3p, Mak10p, and Mak31p. *J Biol Chem*. 276:20154-9.

Portnoy, M.E., X.F. Liu, and V.C. Culotta. 2000. Saccharomyces cerevisiae expresses three functionally distinct homologues of the nramp family of metal transporters. *Mol Cell Biol*. 20:7893-902.

Preisig-Muller, R., G. Schlichthorl, T. Goerge, S. Heinen, A. Bruggemann, S. Rajan, C. Derst, R.W. Veh, and J. Daut. 2002. Heteromerization of Kir2.x potassium channels contributes to the phenotype of Andersen's syndrome. *Proc Natl Acad Sci U S A*. 99:7774-9.

Prince, L.S., and M.J. Welsh. 1998. Cell surface expression and biosynthesis of epithelial Na+ channels. *Biochem J*. 336 (Pt 3):705-10.

Priori, S.G., S.V. Pandit, I. Rivolta, O. Berenfeld, E. Ronchetti, A. Dhamoon, C. Napolitano, J. Anumonwo, M.R. di Barletta, S. Gudapakkam, G. Bosi, M. Stramba-Badiale, and J. Jalife. 2005. A novel form of short QT syndrome (SQT3) is caused by a mutation in the KCNJ2 gene. *Circ Res*. 96:800-7.

Qu, D., J.H. Teckman, S. Omura, and D.H. Perlmutter. 1996. Degradation of a mutant secretory protein, alpha1-antitrypsin Z, in the endoplasmic reticulum requires proteasome activity. *J Biol Chem*. 271:22791-5.

Rabinovich, E., A. Kerem, K.U. Frohlich, N. Diamant, and S. Bar-Nun. 2002. AAA-ATPase p97/Cdc48p, a cytosolic chaperone required for endoplasmic reticulum-associated protein degradation. *Mol Cell Biol*. 22:626-34.

Raiborg, C., B. Bremnes, A. Mehlum, D.J. Gillooly, A. D'Arrigo, E. Stang, and H. Stenmark. 2001. FYVE and coiled-coil domains determine the specific localisation of Hrs to early endosomes. *J Cell Sci*. 114:2255-63.

Ram, R.J., B. Li, and C.A. Kaiser. 2002. Identification of Sec36p, Sec37p, and Sec38p: components of yeast complex that contains Sec34p and Sec35p. *Mol Biol Cell*. 13:1484-500.

Rampoldi, L., C. Dobson-Stone, J.P. Rubio, A. Danek, R.M. Chalmers, N.W. Wood, C. Verellen, X. Ferrer, A. Malandrini, G.M. Fabrizi, R. Brown, J. Vance, M. Pericak-Vance, G. Rudolf, S. Carre, E. Alonso, M. Manfredi, A.H. Nemeth, and A.P. Monaco. 2001. A conserved sorting-associated protein is mutant in chorea-acanthocytosis. *Nat Genet*. 28:119-20.

Rauh, R., A. Diakov, A. Tzschoppe, J. Korbmacher, A.K. Azad, H. Cuppens, J.J. Cassiman, J. Dotsch, H. Sticht, and C. Korbmacher. 2010. A mutation of the epithelial sodium channel associated with atypical cystic fibrosis increases channel open probability and reduces Na+ self inhibition. *J Physiol*. 588:1211-25.

Raymond, C.K., I. Howald-Stevenson, C.A. Vater, and T.H. Stevens. 1992. Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E vps mutants. *Mol Biol Cell*. 3:1389-402.

Redding, K., J.H. Brickner, L.G. Marschall, J.W. Nichols, and R.S. Fuller. 1996. Allele-specific suppression of a defective trans-Golgi network (TGN) localization signal in Kex2p identifies three genes involved in localization of TGN transmembrane proteins. *Mol Cell Biol*. 16:6208-17.

Reggiori, F., and H.R. Pelham. 2002. A transmembrane ubiquitin ligase required to sort membrane proteins into multivesicular bodies. *Nat Cell Biol*. 4:117-23.

Reinders, J., R.P. Zahedi, N. Pfanner, C. Meisinger, and A. Sickmann. 2006. Toward the complete yeast mitochondrial proteome: multidimensional separation techniques for mitochondrial proteomics. *J Proteome Res*. 5:1543-54.

Richly, H., M. Rape, S. Braun, S. Rumpf, C. Hoege, and S. Jentsch. 2005. A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. *Cell*. 120:73-84.

Roberg, K.J., N. Rowley, and C.A. Kaiser. 1997. Physiological regulation of membrane protein sorting late in the secretory pathway of Saccharomyces cerevisiae. *J Cell Biol*. 137:1469-82.

Robinson, J.S., D.J. Klionsky, L.M. Banta, and S.D. Emr. 1988. Protein sorting in Saccharomyces cerevisiae: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. *Mol Cell Biol*. 8:4936-48.

Romanenko, V.G., Y. Fang, F. Byfield, A.J. Travis, C.A. Vandenberg, G.H. Rothblat, and I. Levitan. 2004. Cholesterol sensitivity and lipid raft targeting of Kir2.1 channels. *Biophys J*. 87:3850-61.

Rosenwald, A.G., M.A. Rhodes, H. Van Valkenburgh, V. Palanivel, G. Chapman, A. Boman, C.J. Zhang, and R.A. Kahn. 2002. ARL1 and membrane traffic in Saccharomyces cerevisiae. *Yeast*. 19:1039-56.

Rothman, J.H., I. Howald, and T.H. Stevens. 1989. Characterization of genes required for protein sorting and vacuolar function in the yeast Saccharomyces cerevisiae. *EMBO J*. 8:2057-65.

Rothman, J.H., and T.H. Stevens. 1986. Protein sorting in yeast: mutants defective in vacuole biogenesis mislocalize vacuolar proteins into the late secretory pathway. *Cell*. 47:1041-51.

Rotin, D., V. Kanelis, and L. Schild. 2001. Trafficking and cell surface stability of ENaC. *Am J Physiol Renal Physiol*. 281:F391-9.

Roumanie, O., C. Weinachter, I. Larrieu, M. Crouzet, and F. Doignon. 2001. Functional characterization of the Bag7, Lrg1 and Rgd2 RhoGAP proteins from Saccharomyces cerevisiae. *FEBS Lett*. 506:149-56.

Rue, S.M., S. Mattei, S. Saksena, and S.D. Emr. 2008. Novel Ist1-Did2 complex functions at a late step in multivesicular body sorting. *Mol Biol Cell*. 19:475-84.

Ruknudin, A., D.H. Schulze, S.K. Sullivan, W.J. Lederer, and P.A. Welling. 1998. Novel subunit composition of a renal epithelial KATP channel. *J Biol Chem*. 273:14165-71.

Rusten, T.E., and H. Stenmark. 2009. How do ESCRT proteins control autophagy? *J Cell Sci*. 122:2179-83.

Sacher, M., J. Barrowman, D. Schieltz, J.R. Yates, 3rd, and S. Ferro-Novick. 2000. Identification and characterization of five new subunits of TRAPP. *Eur J Cell Biol*. 79:71-80.

Saksena, S., and S.D. Emr. 2009. ESCRTs and human disease. *Biochem Soc Trans*. 37:167-72.

Samson, R.Y., T. Obita, S.M. Freund, R.L. Williams, and S.D. Bell. 2008. A role for the ESCRT system in cell division in archaea. *Science*. 322:1710-3.

Sarthy, A.V., T. McGonigal, M. Coen, D.J. Frost, J.A. Meulbroek, and R.C. Goldman. 1997. Phenotype in Candida albicans of a disruption of the BGL2 gene encoding a 1,3-beta-glucosyltransferase. *Microbiology*. 143 (Pt 2):367-76.

Sato, B.K., D. Schulz, P.H. Do, and R.Y. Hampton. 2009. Misfolded membrane proteins are specifically recognized by the transmembrane domain of the Hrd1p ubiquitin ligase. *Mol Cell*. 34:212-22.

Scheiner-Bobis, G. 2001. Sanguinarine induces K+ outflow from yeast cells expressing mammalian sodium pumps. *Naunyn Schmiedebergs Arch Pharmacol*. 363:203-8.

Scheiner-Bobis, G., and R.A. Farley. 1994. Subunit requirements for expression of functional sodium pumps in yeast cells. *Biochim Biophys Acta*. 1193:226-34.

Scheiner-Bobis, G., D. Meyer zu Heringdorf, M. Christ, and E. Habermann. 1994. Palytoxin induces K+ efflux from yeast cells expressing the mammalian sodium pump. *Mol Pharmacol*. 45:1132-6.

Scheiner-Bobis, G., and H. Schneider. 1997. Palytoxin-induced channel formation within the Na+/K+-ATPase does not require a catalytically active enzyme. *Eur J Biochem*. 248:717-23.

Scheiner-Bobis, G., and S. Schreiber. 1999. Glutamic acid 472 and lysine 480 of the sodium pump alpha 1 subunit are essential for activity. Their conservation in pyrophosphatases suggests their involvement in recognition of ATP phosphates. *Biochemistry*. 38:9198-208.

Schmidt, A.E., T. Miller, S.L. Schmidt, R. Shiekhattar, and A. Shilatifard. 1999. Cloning and characterization of the EAP30 subunit of the ELL complex that confers derepression of transcription by RNA polymerase II. *J Biol Chem*. 274:21981-5.

Scholl, U.I., M. Choi, T. Liu, V.T. Ramaekers, M.G. Hausler, J. Grimmer, S.W. Tobe, A. Farhi, C. Nelson-Williams, and R.P. Lifton. 2009. Seizures, sensorineural deafness, ataxia, mental retardation, and electrolyte imbalance (SeSAME syndrome) caused by mutations in KCNJ10. *Proc Natl Acad Sci U S A*. 106:5842-7.

Schubert, U., L.C. Anton, I. Bacik, J.H. Cox, S. Bour, J.R. Bennink, M. Orlowski, K. Strebel, and J.W. Yewdell. 1998. CD4 glycoprotein degradation induced by human immunodeficiency virus type 1 Vpu protein requires the function of proteasomes and the ubiquitin-conjugating pathway. *J Virol*. 72:2280-8.

Scrivens, P.J., N. Shahrzad, A. Moores, A. Morin, S. Brunet, and M. Sacher. 2009. TRAPPC2L is a novel, highly conserved TRAPP-interacting protein. *Traffic*. 10:724-36.

Seaman, M.N. 2008. Endosome protein sorting: motifs and machinery. *Cell Mol Life Sci*. 65:2842-58.

Seaman, M.N., E.G. Marcusson, J.L. Cereghino, and S.D. Emr. 1997. Endosome to Golgi retrieval of the vacuolar protein sorting receptor, Vps10p, requires the function of the VPS29, VPS30, and VPS35 gene products. *J Cell Biol*. 137:79-92.

Seaman, M.N., J.M. McCaffery, and S.D. Emr. 1998. A membrane coat complex essential for endosome-to-Golgi retrograde transport in yeast. *J Cell Biol*. 142:665-81.

Sekiguchi, T., E. Hirose, N. Nakashima, M. Ii, and T. Nishimoto. 2001. Novel G proteins, Rag C and Rag D, interact with GTP-binding proteins, Rag A and Rag B. *J Biol Chem*. 276:7246-57.

Seol, J.H., A. Shevchenko, and R.J. Deshaies. 2001. Skp1 forms multiple protein complexes, including RAVE, a regulator of V-ATPase assembly. *Nat Cell Biol*. 3:384-91.

Serohijos, A.W., T. Hegedus, A.A. Aleksandrov, L. He, L. Cui, N.V. Dokholyan, and J.R. Riordan. 2008. Phenylalanine-508 mediates a cytoplasmic-membrane domain contact in the CFTR 3D structure crucial to assembly and channel function. *Proc Natl Acad Sci U S A*. 105:3256-61.

Shcherbik, N., T. Zoladek, J.T. Nickels, and D.S. Haines. 2003. Rsp5p is required for ER bound Mga2p120 polyubiquitination and release of the processed/tethered transactivator Mga2p90. *Curr Biol*. 13:1227-33.

Sheng, S., J. Li, K.A. McNulty, D. Avery, and T.R. Kleyman. 2000. Characterization of the selectivity filter of the epithelial sodium channel. *J Biol Chem*. 275:8572-81.

Shields, S.B., A.J. Oestreich, S. Winistorfer, D. Nguyen, J.A. Payne, D.J. Katzmann, and R. Piper. 2009. ESCRT ubiquitin-binding domains function cooperatively during MVB cargo sorting. *J Cell Biol*. 185:213-24.

Shiflett, S.L., D.M. Ward, D. Huynh, M.B. Vaughn, J.C. Simmons, and J. Kaplan. 2004. Characterization of Vta1p, a class E Vps protein in Saccharomyces cerevisiae. *J Biol Chem*. 279:10982-90.

Shimizu, J., K. Yoda, and M. Yamasaki. 1994. The hypo-osmolarity-sensitive phenotype of the Saccharomyces cerevisiae hpo2 mutant is due to a mutation in PKC1, which regulates expression of beta-glucanase. *Mol Gen Genet*. 242:641-8.

Shinbo, I., K. Fushimi, M. Kasahara, K. Yamauchi, S. Sasaki, and F. Marumo. 1999. Functional analysis of aquaporin-2 mutants associated with nephrogenic diabetes insipidus by yeast expression. *Am J Physiol*. 277:F734-41.

Simonin, A., and D. Fuster. 2010. Nedd4-1 and beta-arrestin-1 are key regulators of Na+/H+ exchanger 1 ubiquitylation, endocytosis, and function. *J Biol Chem*. 285:38293-303.

Siniossoglou, S., and H.R. Pelham. 2002. Vps51p links the VFT complex to the SNARE Tlg1p. *J Biol Chem*. 277:48318-24.

Sipos, G., J.H. Brickner, E.J. Brace, L. Chen, A. Rambourg, F. Kepes, and R.S. Fuller. 2004. Soi3p/Rav1p functions at the early endosome to regulate endocytic trafficking to the vacuole and localization of trans-Golgi network transmembrane proteins. *Mol Biol Cell*. 15:3196-209.

Skibinski, G., N.J. Parkinson, J.M. Brown, L. Chakrabarti, S.L. Lloyd, H. Hummerich, J.E. Nielsen, J.R. Hodges, M.G. Spillantini, T. Thusgaard, S. Brandner, A. Brun, M.N. Rossor, A. Gade, P. Johannsen, S.A. Sorensen, S. Gydesen, E.M. Fisher, and J. Collinge. 2005. Mutations in the endosomal ESCRTIII-complex subunit CHMP2B in frontotemporal dementia. *Nat Genet*. 37:806-8.

Smith, M.H., H.L. Ploegh, and J.S. Weissman. 2011. Road to ruin: targeting proteins for degradation in the endoplasmic reticulum. *Science*. 334:1086-90.

Snyder, P.M. 2002. The epithelial Na+ channel: cell surface insertion and retrieval in Na+ homeostasis and hypertension. *Endocr Rev*. 23:258-75.

Snyder, P.M. 2005. Minireview: regulation of epithelial Na+ channel trafficking. *Endocrinology*. 146:5079-85.

Snyder, P.M., D.R. Olson, and B.C. Thomas. 2002. Serum and glucocorticoid-regulated kinase modulates Nedd4-2-mediated inhibition of the epithelial Na+ channel. *J Biol Chem*. 277:5-8.

Soulsby, N., H. Greville, K. Coulthard, and C. Doecke. 2009. Renal dysfunction in cystic fibrosis: is there cause for concern? *Pediatr Pulmonol*. 44:947-53.

Soundararajan, R., D. Pearce, R.P. Hughey, and T.R. Kleyman. 2010. Role of epithelial sodium channels and their regulators in hypertension. *J Biol Chem*. 285:30363-9.

Spasser, L., and A. Brik. 2012. Chemistry and biology of the ubiquitin signal. *Angew Chem Int Ed Engl*. 51:6840-62.

Spear, E.D., and D.T. Ng. 2003. Stress tolerance of misfolded carboxypeptidase Y requires maintenance of protein trafficking and degradative pathways. *Mol Biol Cell*. 14:2756-67.

Staub, O., I. Gautschi, T. Ishikawa, K. Breitschopf, A. Ciechanover, L. Schild, and D. Rotin. 1997. Regulation of stability and function of the epithelial Na+ channel (ENaC) by ubiquitination. *EMBO J*. 16:6325-36.

Stefansson, B., and D.L. Brautigan. 2006. Protein phosphatase 6 subunit with conserved Sit4-associated protein domain targets IkappaBepsilon. *J Biol Chem*. 281:22624-34.

Steffensen, L., and P.A. Pedersen. 2003. Responses at the translational level to heterologous expression of the Na,K-ATPase. *Ann N Y Acad Sci*. 986:539-40.

Steffensen, L., and P.A. Pedersen. 2006. Heterologous expression of membrane and soluble proteins derepresses GCN4 mRNA translation in the yeast Saccharomyces cerevisiae. *Eukaryot Cell*. 5:248-61.

Stewart, A.P., S. Haerteis, A. Diakov, C. Korbmacher, and J.M. Edwardson. 2011. Atomic force microscopy reveals the architecture of the epithelial sodium channel (ENaC). *J Biol Chem*. 286:31944-52.

Stimpson, H.E., M.J. Lewis, and H.R. Pelham. 2006. Transferrin receptor-like proteins control the degradation of a yeast metal transporter. *EMBO J*. 25:662-72.

Stockand, J.D., A. Staruschenko, O. Pochynyuk, R.E. Booth, and D.U. Silverthorn. 2008. Insight toward epithelial Na+ channel mechanism revealed by the acid-sensing ion channel 1 structure. *IUBMB Life*. 60:620-8.

Stockklausner, C., and N. Klocker. 2003. Surface expression of inward rectifier potassium channels is controlled by selective Golgi export. *J Biol Chem*. 278:17000-5.

Stockklausner, C., J. Ludwig, J.P. Ruppersberg, and N. Klocker. 2001. A sequence motif responsible for ER export and surface expression of Kir2.0 inward rectifier K(+) channels. *FEBS Lett*. 493:129-33.

Stolz, A., W. Hilt, A. Buchberger, and D.H. Wolf. 2011. Cdc48: a power machine in protein degradation. *Trends Biochem Sci*. 36:515-23.

Su, P., and G. Scheiner-Bobis. 2004. Lys691 and Asp714 of the Na+/K+-ATPase alpha subunit are essential for phosphorylation, dephosphorylation, and enzyme turnover. *Biochemistry*. 43:4731-40.

Su, Y.R., and A.G. Menon. 2001. Epithelial sodium channels and hypertension. *Drug Metab Dispos*. 29:553-6.

Sullivan, M.L., R.T. Youker, S.C. Watkins, and J.L. Brodsky. 2003. Localization of the BiP molecular chaperone with respect to endoplasmic reticulum foci containing the cystic fibrosis transmembrane conductance regulator in yeast. *J Histochem Cytochem*. 51:545-8.

Sun, Y., R. Taniguchi, D. Tanoue, T. Yamaji, H. Takematsu, K. Mori, T. Fujita, T. Kawasaki, and Y. Kozutsumi. 2000. Sli2 (Ypk1), a homologue of mammalian protein kinase SGK, is a downstream kinase in the sphingolipid-mediated signaling pathway of yeast. *Mol Cell Biol*. 20:4411-9.

Szabo, A., T. Langer, H. Schroder, J. Flanagan, B. Bukau, and F.U. Hartl. 1994. The ATP hydrolysis-dependent reaction cycle of the Escherichia coli Hsp70 system DnaK, DnaJ, and GrpE. *Proc Natl Acad Sci U S A*. 91:10345-9.

Tamarappoo, B.K., B. Yang, and A.S. Verkman. 1999. Misfolding of mutant aquaporin-2 water channels in nephrogenic diabetes insipidus. *J Biol Chem*. 274:34825-31.

Tang, W., C.L. Qin, and X.C. Yang. 1995a. Cloning, localization, and functional expression of a human brain inward rectifier potassium channel (hIRK1). *Receptors Channels*. 3:175-83.

Tang, W., A. Ruknudin, W.P. Yang, S.Y. Shaw, A. Knickerbocker, and S. Kurtz. 1995b. Functional expression of a vertebrate inwardly rectifying K+ channel in yeast. *Mol Biol Cell*. 6:1231-40.

Taxis, C., F. Vogel, and D.H. Wolf. 2002. ER-golgi traffic is a prerequisite for efficient ER degradation. *Mol Biol Cell*. 13:1806-18.

Teem, J.L., H.A. Berger, L.S. Ostedgaard, D.P. Rich, L.C. Tsui, and M.J. Welsh. 1993. Identification of revertants for the cystic fibrosis delta F508 mutation using STE6-CFTR chimeras in yeast. *Cell*. 73:335-46.

Teem, J.L., M.R. Carson, and M.J. Welsh. 1996. Mutation of R555 in CFTR-delta F508 enhances function and partially corrects defective processing. *Receptors Channels*. 4:63-72.

Teo, H., D.J. Gill, J. Sun, O. Perisic, D.B. Veprintsev, Y. Vallis, S.D. Emr, and R.L. Williams. 2006. ESCRT-I core and ESCRT-II GLUE domain structures reveal role for GLUE in linking to ESCRT-I and membranes. *Cell*. 125:99-111.

Teo, H., O. Perisic, B. Gonzalez, and R.L. Williams. 2004. ESCRT-II, an endosome-associated complex required for protein sorting: crystal structure and interactions with ESCRT-III and membranes. *Dev Cell*. 7:559-69.

Thrower, J.S., L. Hoffman, M. Rechsteiner, and C.M. Pickart. 2000. Recognition of the polyubiquitin proteolytic signal. *EMBO J*. 19:94-102.

Tinker, A., Y.N. Jan, and L.Y. Jan. 1996. Regions responsible for the assembly of inwardly rectifying potassium channels. *Cell*. 87:857-68.

Tong, A.H., and C. Boone. 2006. Synthetic genetic array analysis in Saccharomyces cerevisiae. *Methods Mol Biol*. 313:171-92.

Tong, A.H., and C. Boone. 2007. High-Throughput Strain Construction and Systematic Synthetic Lethal Screening in *Saccharomycs cerevisiae. In* Yeast Gene Analysis. Vol. 36. I. Stansfield and M.J.R. Stark, editors. Elsevier Ltd. 369-386, 706-707.

Tong, Y., G.S. Brandt, M. Li, G. Shapovalov, E. Slimko, A. Karschin, D.A. Dougherty, and H.A. Lester. 2001. Tyrosine decaging leads to substantial membrane trafficking during modulation of an inward rectifier potassium channel. *J Gen Physiol*. 117:103-18.

Trzcinska-Daneluti, A.M., D. Ly, L. Huynh, C. Jiang, C. Fladd, and D. Rotin. 2009. High-content functional screen to identify proteins that correct F508del-CFTR function. *Mol Cell Proteomics*. 8:780-90.

Tsang, H.T., J.W. Connell, S.E. Brown, A. Thompson, E. Reid, and C.M. Sanderson. 2006. A systematic analysis of human CHMP protein interactions: additional MIT domain-containing proteins bind to multiple components of the human ESCRT III complex. *Genomics*. 88:333-46.

Tsukada, M., and Y. Ohsumi. 1993. Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae. *FEBS Lett*. 333:169-74.

Ungar, D., T. Oka, E.E. Brittle, E. Vasile, V.V. Lupashin, J.E. Chatterton, J.E. Heuser, M. Krieger, and M.G. Waters. 2002. Characterization of a mammalian Golgi-localized protein complex, COG, that is required for normal Golgi morphology and function. *J Cell Biol*. 157:405-15.

Vaidyanathan, R., S.M. Taffet, K.L. Vikstrom, and J.M. Anumonwo. 2010. Regulation of cardiac inward rectifier potassium current (I(K1)) by synapse-associated protein-97. *J Biol Chem*. 285:28000-9.

Vainberg, I.E., S.A. Lewis, H. Rommelaere, C. Ampe, J. Vandekerckhove, H.L. Klein, and N.J. Cowan. 1998. Prefoldin, a chaperone that delivers unfolded proteins to cytosolic chaperonin. *Cell*. 93:863-73.

Valentijn, J.A., G.K. Fyfe, and C.M. Canessa. 1998. Biosynthesis and processing of epithelial sodium channels in Xenopus oocytes. *J Biol Chem*. 273:30344-51.

Vallejo, A.N., R.J. Pogulis, and L.R. Pease. 1994. In vitro synthesis of novel genes: mutagenesis and recombination by PCR. *PCR Methods Appl*. 4:S123-30.

Vallier, L.G., and M. Carlson. 1991. New SNF genes, GAL11 and GRR1 affect SUC2 expression in Saccharomyces cerevisiae. *Genetics*. 129:675-84.

VanSlyke, J.K., S.M. Deschenes, and L.S. Musil. 2000. Intracellular transport, assembly, and degradation of wild-type and disease-linked mutant gap junction proteins. *Mol Biol Cell*. 11:1933-46.

Vashist, S., W. Kim, W.J. Belden, E.D. Spear, C. Barlowe, and D.T. Ng. 2001. Distinct retrieval and retention mechanisms are required for the quality control of endoplasmic reticulum protein folding. *J Cell Biol*. 155:355-68.

Vashist, S., and D.T. Ng. 2004. Misfolded proteins are sorted by a sequential checkpoint mechanism of ER quality control. *J Cell Biol*. 165:41-52.

Velayos-Baeza, A., A. Vettori, R.R. Copley, C. Dobson-Stone, and A.P. Monaco. 2004. Analysis of the human VPS13 gene family. *Genomics*. 84:536-49.

Vembar, S.S., and J.L. Brodsky. 2008. One step at a time: endoplasmic reticulum-associated degradation. *Nat Rev Mol Cell Biol*. 9:944-57.

Vidal, M., A.M. Buckley, F. Hilger, and R.F. Gaber. 1990. Direct selection for mutants with increased K+ transport in Saccharomyces cerevisiae. *Genetics*. 125:313-20.

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

Virkki, L.V., J. Biber, H. Murer, and I.C. Forster. 2007. Phosphate transporters: a tale of two solute carrier families. *Am J Physiol Renal Physiol*. 293:F643-54.

Vollmer, P., E. Will, D. Scheglmann, M. Strom, and D. Gallwitz. 1999. Primary structure and biochemical characterization of yeast GTPase-activating proteins with substrate preference for the transport GTPase Ypt7p. *Eur J Biochem*. 260:284-90.

Wang, G., J. Yang, and J.M. Huibregtse. 1999. Functional domains of the Rsp5 ubiquitin-protein ligase. *Mol Cell Biol*. 19:342-52.

Wang, M., M. Weiss, M. Simonovic, G. Haertinger, S.P. Schrimpf, M.O. Hengartner, and C. von Mering. 2012. PaxDb, a database of protein abundance averages across all three domains of life. *Mol Cell Proteomics*. 11:492-500.

Wang, Q., and A. Chang. 2003. Substrate recognition in ER-associated degradation mediated by Eps1, a member of the protein disulfide isomerase family. *EMBO J*. 22:3792-802.

Wang, S., and D.T. Ng. 2010. Evasion of endoplasmic reticulum surveillance makes Wsc1p an obligate substrate of Golgi quality control. *Mol Biol Cell*. 21:1153-65.

Wang, S., G. Thibault, and D.T. Ng. 2011. Routing misfolded proteins through the multivesicular body (MVB) pathway protects against proteotoxicity. *J Biol Chem*. 286:29376-87.

Wang, S.G., and R.A. Farley. 1998. Valine 904, tyrosine 898, and cysteine 908 in Na,K-ATPase alpha subunits are important for assembly with beta subunits. *J Biol Chem*. 273:29400-5.

Wang, T.F., J.H. Chang, and C. Wang. 1993. Identification of the peptide binding domain of hsc70. 18-Kilodalton fragment located immediately after ATPase domain is sufficient for high affinity binding. *J Biol Chem*. 268:26049-51.

Wang, X., J. Venable, P. LaPointe, D.M. Hutt, A.V. Koulov, J. Coppinger, C. Gurkan, W. Kellner, J. Matteson, H. Plutner, J.R. Riordan, J.W. Kelly, J.R. Yates, 3rd, and W.E. Balch. 2006. Hsp90 cochaperone Aha1 downregulation rescues misfolding of CFTR in cystic fibrosis. *Cell*. 127:803-15.

Ward, C.L., S. Omura, and R.R. Kopito. 1995. Degradation of CFTR by the ubiquitin-proteasome pathway. *Cell*. 83:121-7.

Wassmer, T., N. Attar, M. Harterink, J.R. van Weering, C.J. Traer, J. Oakley, B. Goud, D.J. Stephens, P. Verkade, H.C. Korswagen, and P.J. Cullen. 2009. The retromer coat complex coordinates endosomal sorting and dynein-mediated transport, with carrier recognition by the trans-Golgi network. *Dev Cell*. 17:110-22.

Webster, G., and C.I. Berul. 2013. An update on channelopathies: from mechanisms to management. *Circulation*. 127:126-40.

Wehman, A.M., C. Poggioli, P. Schweinsberg, B.D. Grant, and J. Nance. 2011. The P4-ATPase TAT-5 inhibits the budding of extracellular vesicles in C. elegans embryos. *Curr Biol*. 21:1951-9.

Weisz, O.A., and J.P. Johnson. 2003. Noncoordinate regulation of ENaC: paradigm lost? *Am J Physiol Renal Physiol*. 285:F833-42.

Wiemuth, D., Y. Ke, M. Rohlfs, and F.J. McDonald. 2007. Epithelial sodium channel (ENaC) is multi-ubiquitinated at the cell surface. *Biochem J*. 405:147-55.

Winderickx, J., C. Delay, A. De Vos, H. Klinger, K. Pellens, T. Vanhelmont, F. Van Leuven, and P. Zabrocki. 2008. Protein folding diseases and neurodegeneration: lessons learned from yeast. *Biochim Biophys Acta*. 1783:1381-95.

Winzeler, E.A., D.D. Shoemaker, A. Astromoff, H. Liang, K. Anderson, B. Andre, R. Bangham, R. Benito, J.D. Boeke, H. Bussey, A.M. Chu, C. Connelly, K. Davis, F. Dietrich, S.W. Dow, M. El Bakkoury, F. Foury, S.H. Friend, E. Gentalen, G. Giaever, J.H. Hegemann, T. Jones, M. Laub, H. Liao, N. Liebundguth, D.J. Lockhart, A. Lucau-Danila, M. Lussier, N. M'Rabet, P. Menard, M. Mittmann, C. Pai, C. Rebischung, J.L. Revuelta, L. Riles, C.J. Roberts, P. Ross-MacDonald, B. Scherens, M. Snyder, S. Sookhai-Mahadeo, R.K. Storms, S. Veronneau, M. Voet, G. Volckaert, T.R. Ward, R. Wysocki, G.S. Yen, K. Yu, K. Zimmermann, P. Philippsen, M. Johnston, and R.W. Davis. 1999. Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. *Science*. 285:901-6.

Wischmeyer, E., F. Doring, and A. Karschin. 1998. Acute suppression of inwardly rectifying Kir2.1 channels by direct tyrosine kinase phosphorylation. *J Biol Chem*. 273:34063-8.

Wolf, D.H., and A. Stolz. 2012. The Cdc48 machine in endoplasmic reticulum associated protein degradation. *Biochim Biophys Acta*. 1823:117-24.

Wollert, T., and J.H. Hurley. 2010. Molecular mechanism of multivesicular body biogenesis by ESCRT complexes. *Nature*. 464:864-9.

Wollert, T., C. Wunder, J. Lippincott-Schwartz, and J.H. Hurley. 2009. Membrane scission by the ESCRT-III complex. *Nature*. 458:172-7.

Wright, M.B., E.A. Howell, and R.F. Gaber. 1996. Amino acid substitutions in membrane-spanning domains of Hol1, a member of the major facilitator superfamily of transporters, confer nonselective cation uptake in Saccharomyces cerevisiae. *J Bacteriol*. 178:7197-205.

Xia, W.F., F.L. Tang, L. Xiong, S. Xiong, J.U. Jung, D.H. Lee, X.S. Li, X. Feng, L. Mei, and W.C. Xiong. 2013. Vps35 loss promotes hyperresorptive osteoclastogenesis and osteoporosis via sustained RANKL signaling. *J Cell Biol*. 200:821-37.

Xiang, M., M. Feng, S. Muend, and R. Rao. 2007. A human Na+/H+ antiporter sharing evolutionary origins with bacterial NhaA may be a candidate gene for essential hypertension. *Proc Natl Acad Sci U S A*. 104:18677-81.

Xie, W., L. Li, and S.N. Cohen. 1998. Cell cycle-dependent subcellular localization of the TSG101 protein and mitotic and nuclear abnormalities associated with TSG101 deficiency. *Proc Natl Acad Sci U S A*. 95:1595-600.

Xu, G., R.A. Farley, D.J. Kane, and L.D. Faller. 2003a. Site-directed mutagenesis of amino acids in the cytoplasmic loop 6/7 of Na,K-ATPase. *Ann N Y Acad Sci*. 986:96-100.

Xu, Z., L. Liang, H. Wang, T. Li, and M. Zhao. 2003b. HCRP1, a novel gene that is downregulated in hepatocellular carcinoma, encodes a growth-inhibitory protein. *Biochem Biophys Res Commun*. 311:1057-66.

Ye, Y., H.H. Meyer, and T.A. Rapoport. 2001. The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature*. 414:652-6.

Yeo, S.C., L. Xu, J. Ren, V.J. Boulton, M.D. Wagle, C. Liu, G. Ren, P. Wong, R. Zahn, P. Sasajala, H. Yang, R.C. Piper, and A.L. Munn. 2003. Vps20p and Vta1p interact with Vps4p and function in multivesicular body sorting and endosomal transport in Saccharomyces cerevisiae. *J Cell Sci*. 116:3957-70.

Yoo, D., L. Fang, A. Mason, B.Y. Kim, and P.A. Welling. 2005. A phosphorylation-dependent export structure in ROMK (Kir 1.1) channel overrides an endoplasmic reticulum localization signal. *J Biol Chem*. 280:35281-9.

Yoon, T., and K. Lee. 1998. Isoform-specific interaction of the cytoplasmic domains of Na,K-ATPase. *Mol Cells*. 8:606-13.

Youker, R.T., P. Walsh, T. Beilharz, T. Lithgow, and J.L. Brodsky. 2004. Distinct roles for the Hsp40 and Hsp90 molecular chaperones during cystic fibrosis transmembrane conductance regulator degradation in yeast. *Mol Biol Cell*. 15:4787-97.

Yuan, H., K. Michelsen, and B. Schwappach. 2003. 14-3-3 dimers probe the assembly status of multimeric membrane proteins. *Curr Biol*. 13:638-46.

Yukutake, Y., S. Tsuji, Y. Hirano, T. Adachi, T. Takahashi, K. Fujihara, P. Agre, M. Yasui, and M. Suematsu. 2008. Mercury chloride decreases the water permeability of aquaporin-4-reconstituted proteoliposomes. *Biol Cell*. 100:355-63.

Zaks-Makhina, E., Y. Kim, E. Aizenman, and E.S. Levitan. 2004. Novel neuroprotective K+ channel inhibitor identified by high-throughput screening in yeast. *Mol Pharmacol*. 65:214-9.

Zaks-Makhina, E., H. Li, A. Grishin, V. Salvador-Recatala, and E.S. Levitan. 2009. Specific and slow inhibition of the kir2.1 K+ channel by gambogic acid. *J Biol Chem*. 284:15432-8.

Zeidel, M., E. Rey, J. Tami, M. Fischbach, and I. Sanz. 1995. Genetic and functional characterization of human autoantibodies using combinatorial phage display libraries. *Ann N Y Acad Sci*. 764:559-64.

Zhang, X.M., B. Walsh, C.A. Mitchell, and T. Rowe. 2005. TBC domain family, member 15 is a novel mammalian Rab GTPase-activating protein with substrate preference for Rab7. *Biochem Biophys Res Commun*. 335:154-61.

Zhang, Y., G. Nijbroek, M.L. Sullivan, A.A. McCracken, S.C. Watkins, S. Michaelis, and J.L. Brodsky. 2001. Hsp70 molecular chaperone facilitates endoplasmic reticulum-associated protein degradation of cystic fibrosis transmembrane conductance regulator in yeast. *Mol Biol Cell*. 12:1303-14.

Zhong, Y., and S. Fang. 2012. Live cell imaging of protein dislocation from the endoplasmic reticulum. *J Biol Chem*. 287:28057-66.

Zhou, R., S.V. Patel, and P.M. Snyder. 2007. Nedd4-2 catalyzes ubiquitination and degradation of cell surface ENaC. *J Biol Chem*. 282:20207-12.