Development of Fluorogen Activating Protein Tags for Quantitative Trafficking Studies of the Mammalian Potassium Channel Kir2.1 in *Saccharomyces cerevisiae*

by

Natalie Anne Hager

Bachelor of Science, Juniata College, 2016

Submitted to the Graduate Faculty of the Dietrich School of Arts and Sciences in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2022
This dissertation was presented

by

Natalie Anne Hager

It was defended on

July 5, 2022

and approved by

Jon P. Boyle, Ph.D., Associate Professor, Department of Biological Sciences

Jeffery L. Brodsky, Ph.D., Professor, Department of Biological Sciences

Anne E. Carlson Ph.D., Associate Professor, Department of Biological Sciences

Arohan R. Subramanya, M.D., Associate Professor, Department of Medicine

Dissertation Director: Allyson F. O’Donnell, Ph.D., Assistant Professor, Department of Biological Sciences
Development of Fluorogen Activating Protein Tags for Quantitative Trafficking Studies of
the Mammalian Potassium Channel Kir2.1 in *Saccharomyces cerevisiae*

Natalie Anne Hager, Ph.D.

University of Pittsburgh, 2022

Protein composition at the plasma membrane must be tightly regulated for optimal cell health. This involves rapid internalization and selective targeting of proteins to and from the cell surface in response to environmental changes. One category of membrane proteins that are tightly regulated are ion channels, which are dynamically relocalized to or from the plasma membrane in response to physiological changes. This allows organisms to ensure normal ion concentrations. Critical to cardiac function specifically is the localization of Kir2.1, an inward rectifying potassium (K\(_{ir}\)) channel, at the cell surface. Kir2.1 restores the resting membrane potential of cardiac cells after each contraction and readies the cells for the next action potential. Defective Kir2.1 trafficking and activity is associated with diseases, including Andersen-Tawil syndrome, highlighting the importance of understanding Kir2.1 trafficking. To elucidate Kir2.1 trafficking, I used a yeast model system where the endogenous potassium channels were deleted and Kir2.1 was expressed and functionality was confirmed using a serial dilution assay. Specifically, I ectopically expressed Kir2.1 in yeast strains that had their endogenous potassium channels, Trk1 and Trk2, deleted and measured growth on low potassium medium. To further evaluate Kir2.1 trafficking I used fluorogen-activating protein (FAP) tags and quantified its cell surface residency. In this document, I include detailed overview of my research on K\(_{ir}\) channels and how they are protein trafficked. Next, I describe how we identified the role of specific α-arrestins, an emerging class of protein trafficking adaptors, as regulators of Kir2.1 trafficking. My research demonstrates that a
subset of α-arrestins increase intracellular potassium levels by stimulating Kir2.1 trafficking to the cell surface. Following this work, I detail the experiments done to define the trafficking pathways used by the α-arrestin to promote cell surface localization of Kir2.1. Then, I describe the development of the fluorogen-activating protein (FAP) tagging technology and the steps I have taken to make FAP tags more readily available for use in the yeast community. Specifically, I have optimized the tag for expression in yeast, defined optimal imaging conditions, and built a library of FAP-tagged plasmid vectors and proteins to mark specific cellular compartments.
Table of Contents

Preface.............................................................................................................................................. xv

1.0 Introduction.................................................................................................................................. 1

1.1 Kir2.1 function and trafficking ................................................................................................. 1

   1.1.1 KCNJ Family ......................................................................................................................... 3

       1.1.1.1 ...................................................................................................................................... 4

       1.1.1.2 G-Protein-Gated Kir Channels (GIRK1-4/Kir3.1-Kir3.4) .............................................. 7

       1.1.1.3 K_{ATP} Channels (Kir6.1 and Kir6.2) ........................................................................... 9

       1.1.1.4 K^{+} Transport Channels (Kir1.1, Kir4.x, Kir5.1, Kir7.1) ........................................... 10

1.1.2 Regulation of Kir2.x by protein trafficking and its role in disease ...................................... 13

       1.1.2.1 Mapping the Kir2.1 trafficking itinerary: machinery and motifs .................. 13

       1.1.2.2 Disease-linked trafficking mutations in Kir2.1 ......................................................... 20

       1.1.2.3 Kir2.1 trafficking studies in Saccharomyces cerevisiae ........................................... 24

1.2 Protein trafficking and the \( \alpha \)-arrestins .............................................................................. 28

       1.2.1 Role of \( \alpha \)-arrestins in endocytosis in yeast and mammals................................. 28

       1.2.2 Role of \( \alpha \)-arrestins in intracellular sorting and recycling .................................. 31

2.0 Select \( \alpha \)-Arrestins Control the Cell-Surface Abundance of the Mammalian Kir2.1

   Potassium Channel in a Yeast Model............................................................................................ 39

   2.1 Introduction ............................................................................................................................... 39

   2.2 Materials and Methods ........................................................................................................... 41

       2.2.1 Yeast strains and growth conditions ........................................................................... 41

       2.2.2 Plasmids and DNA manipulations ............................................................................. 43
2.2.3 Yeast protein extraction and immunoblot analysis ........................................ 45
2.2.4 Protein stability assays .................................................................................. 46
2.2.5 Biochemical fractionation .............................................................................. 47
2.2.6 Biochemical purification and MS analyses .................................................. 48
2.2.7 Inductively coupled plasma mass spectrometry .......................................... 49
2.2.8 FAP staining and confocal microscopy imaging ........................................... 50
2.2.9 Image quantification and statistical analyses ............................................... 51

2.3 Results .............................................................................................................. 52
2.3.1 A targeted screen identifies select α-arrestins as Kir2.1 regulators .......... 52
2.3.2 FAP-tagged Kir2.1 measures surface channel abundance ....................... 57
2.3.3 α-Arrestins increase Kir2.1 residence at the plasma membrane in a Rsp5-
dependent manner ................................................................................................. 64
2.3.4 α-Arrestins phosphorylation impacts Kir2.1 trafficking ............................. 71

2.4 Discussion ........................................................................................................ 74

3.0 Defining the Intracellular Protein Trafficking Pathways Used by the α-Arrestins .... 79

3.1 Introduction ....................................................................................................... 79
3.1.1 Identifying trafficking factors for α-arrestin-mediated trafficking of Kir2.1
in a yeast model system .......................................................................................... 80

3.2 Materials and Methods .................................................................................... 84
3.2.1 Yeast strains and growth conditions ............................................................ 84
3.2.2 Plasmids and DNA manipulations ............................................................... 88

3.3 Results and Discussion ..................................................................................... 89
3.3.1 Kir2.1 function is regulated by the AP-1 complex ..................................... 89
3.3.2 Kir2.1 function and the AP-2 complex .......................................................... 90
3.3.3 Kir2.1 function is regulated by ESCRT .......................................................... 91
3.3.4 Retromer is a regulator of Kir2.1 ....................................................................... 92
3.3.5 Kir2.1 function is regulated by Vrp1 and End3 ............................................... 93
3.3.6 Aly2-mediated trafficking of Kir2.1 is dependent on Npr1 ......................... 94
3.3.7 GGA regulation of Kir2.1 ................................................................................. 95

4.0 Fluorogen Activating Proteins as a Tool for Quantitative Protein Trafficking

Studies In Yeast ........................................................................................................ 97
4.1 Introduction ........................................................................................................ 97
4.2 Materials and Methods ..................................................................................... 103
  4.2.1 Yeast strains and growth conditions ......................................................... 103
  4.2.2 Plasmids and DNA manipulations ............................................................. 103
  4.2.3 Fluorescence-activating protein (FAP) staining and confocal microscopy
      imaging .............................................................................................................. 105
  4.2.4 Image quantification and statistical analysis ............................................. 106
  4.2.5 Optimizing pH experiments ...................................................................... 107
  4.2.6 Flow cytometry .......................................................................................... 107
  4.2.7 Yeast protein extraction and immunoblot analysis .................................... 108
4.3 RESULTS ........................................................................................................... 109
  4.3.1 Codon optimization of FAP for expression in yeast ................................. 109
  4.3.2 Imaging parameters that influence FAP fluorescence .............................. 113
  4.3.3 FAP is degraded by a vacuolar protease .................................................. 117
  4.3.4 Development of tools with the FAP_{optim} tag ...................................... 119
4.3.5 FAP-tagged cellular markers as tools for co-localization studies in yeast..123

4.4 Discussion .............................................................................................................................................. 126

5.0 Conclusions and Future Directions .................................................................................................................. 129

5.1 Significance of This Study ................................................................................................................................. 129

5.1.1 α-Arrestin-mediated trafficking of Kir2.1 ........................................................................................................ 129

5.1.2 Development of the fluorescent activating protein for yeast trafficking studies
........................................................................................................................................................................ 131

5.1.3 Limitations of this study .................................................................................................................................... 132

5.2 FUTURE DIRECTIONS ..................................................................................................................................... 134

5.2.1 Short term .................................................................................................................................................... 134

5.2.2 Long Term .................................................................................................................................................... 136

Appendix A Exploring the Regulation of Kir2.1 By Mammalian α-Arrestins ............................................... 139

Appendix A.1 Introduction ..................................................................................................................................... 139

Appendix A.2 Materials and Methods .................................................................................................................. 141

Appendix A.3 Results and Discussion .................................................................................................................. 144

Bibliography ........................................................................................................................................................ 149
List of Tables

Table 1. Summary of Kir2.x disease causing mutations linked to protein trafficking........ 27
Table 2. Yeast α-arrestins and their cargo ........................................................................ 37
Table 3. Yeast strains used in this study ........................................................................ 42
Table 4. Plasmids used in Chapter 2 ............................................................................. 43
Table 5. List of gene deletions for targeted screen ......................................................... 83
Table 6. List of strains used in Chapter 3 ....................................................................... 85
Table 7. Plasmids used in Chapter 3 ............................................................................. 89
Table 8. Summary of genetic interactions between trafficking machinery and the α-arrestins in controlling Kir2.1-dependent growth on low potassium ..................................... 96
Table 9. Plasmids used in Chapter 4 ............................................................................. 104
Table 10. List of FAP-tagged cellular markers ............................................................... 124
Table 11. Plasmids used in this study .......................................................................... 143
List of Figures

Figure 1. Kir Family localization.................................................................................................................. 4

Figure 2. Structural mapping and trafficking model of Kir2.1................................................................. 23

Figure 3. α-Arrestin summary......................................................................................................................... 36

Figure 4. α-Arrestins Aly1, Aly2, and Ldb19 promote Kir2.1-dependent growth on low-potassium medium ........................................................................................................................................... 54

Figure 5. Kir2.1 rescues the growth of trk1Δ trk2Δ yeast on low potassium medium ........... 56

Figure 6. The use of a fluorgen-activating protein to report on cellular residence ............. 58

Figure 7. FAP-tagged Kir2.1 can be detected at the cell surface...................................................... 60

Figure 8. The plasma membrane residence and activity of Kir2.1 and FAP-tagged Kir2.1 are regulated similarly ...................................................................................................................................................... 63

Figure 9. α-Arrestin regulation of Kir2.1 requires the Rsp5 ubiquitin ligase ...................... 65

Figure 10. Aly1 and Aly2 are ubiquitinlated at K379 and K92.................................................... 67

Figure 11. Overexpression of Aly1, Aly2, and Ldb19 increases both Kir2.1 residence at the plasma membrane and intracellular potassium ........................................................................................................................................... 68

Figure 12. Calcineurin negatively regulates Aly1-mediated Kir2.1 trafficking to the plasma membrane ...................................................................................................................................................... 73

Figure 13. Schematic representation of trafficking machinery assessed for Kir2.1-dependent trafficking .............................................................................................................................................................................. 83

Figure 14. AP-1 Complex regulates Kir2.1 trafficking ........................................................................ 90

Figure 15. AP-2 complex does not affect Kir2.1 trafficking ....................................................... 91

Figure 16. Kir2.1 is regulated by ESCRT ............................................................................................... 92
Figure 17. Retromer regulates Kir2.1 ................................................................. 93
Figure 18. Inhibition of endocytosis affects Kir2.1 trafficking ................................. 94
Figure 19. Deletion of Npr1 inhibits Aly2-mediated trafficking of Kir2.1 ................. 95
Figure 20. Kir2.1 is regulated by Gga1 .................................................................. 96
Figure 21. Utility of the fluorogen activating protein to visualize proteins of interest... 109
Figure 22. Codon optimization of the FAP tag ....................................................... 112
Figure 23. FAP fluorescence pH optimization ....................................................... 114
Figure 24. Imaging parameters that influence FAP fluorescence ......................... 115
Figure 25. Optimal growth temperature for yeast for FAP imaging ....................... 117
Figure 26. Assessing FAP-tag expression from various promoters ....................... 121
Figure 27. Generation of FAP_optim-tagging vector for expression of fusion proteins.. 122
Figure 28. FAP-tagged cellular markers ............................................................... 125
Figure 29. Mammalian α-arrestins in yeast ......................................................... 144
Figure 30. α-Arrestins in HEK293T cells .............................................................. 145
Figure 31. α-Arrestins in cardiomyocytes ............................................................. 147
List of Abbreviations

Below includes a list of abbreviations used in this dissertation. Common abbreviations used in molecular biology (i.e., DNA, pH) are omitted.

a.u.: Arbitrary units
AP-1: Adaptin Protein complex 1
AP-2: Adaptin Protein complex 2
AzC: Azetidine-2-carboxylic acid
β2AR: β2-adrenergic receptor
BMP: Bone morphogenetic protein
CARC: Cholesterol recognition amino acid consensus motif
CIE: Clathrin-independent endocytosis
CME: Clathrin-mediated endocytosis
COL1A1: Collagen 1 alpha
CFA: Cranial facial abnormality
ER: Endoplasmic Reticulum
ESCRT: Endosomal sorting complex required for transport
ERAD: ER-associated degradation
Dpp: Decapentaplegic
FA: Focal adhesion
FAP: fluorogen-activating protein
GRIF-1: g-aminobutyric acid receptor-interacting factor 1
GABA\(_A\) receptor: \(\gamma\)-aminobutyric acid type A receptor
GRIP: Golgin-97, RanBP2alpha, Imh1p and p230/golgin-245
GST: Glutathione \(S\)-transferase
ICP-MS: Inductively coupled plasma mass spectrometry
LatA: Latrunculin A
MAGUK: Membrane-associated guanylate kinase
MEF2: Myocyte-enhancer factor 2
MG: Malachite green
MSC: Mesenchymal stem cells
MVB: Multivesicular body
Nedd4: neural precursor development downregulated protein 4
OCN: Osteocalcin
PM: Plasma Membrane
PIP\(_2\): Phosphatidylinositol 4,5-bisphosphate
PDZ domain: Post synaptic density, discs large, and zonaula occludens domain
K\(^+\): Potassium
Kir: Potassium Inwardly Rectifying
Retromer: Retrieval of vacuolar-targeted proteins
SC: Synthetic complete
SCA: Single chain antibody
Tkv: Thickveins
TM: Transmembrane
TRAK2: Trafficking kinesin protein 2
TGN: trans-Golgi network
TGF-βs: Transforming growth factor beta
Preface

I remember my first interaction with research very clearly, I was in 5th grade and visiting my sister, Hillary, who at the time was completing her PhD in developmental biology. She took me to her lab where I was introduced to *Xenopus laevis* and told that, from these “tiny dots” in a Petri dish, scientists were able to learn about cell adhesion and vesicular transport. This experience encouraged me to further pursue the sciences and paved the path to my undergraduate studies at Juniata College and then graduate studies at the University of Pittsburgh. So, thank you, Hillary for first igniting my interest in science and continuing to be a source of support and motivation over the years.

I would like to thank my parents – Maureen and Bill Hager – for their unwavering support and encouragement. For knowing that I could always come home and be greeted with homemade cookies, fresh cut fruit, and an invite for a bike ride on the bike trail. I want to thank my siblings – Hillary, Nathan, and Nick – for always being just a phone call away when I needed them. Thank you Nick and Tori for always being down for a walk with Mozzie. As the youngest of the family, I appreciate each of you always opening up your home to me when I wanted to escape the city. I am grateful for the time that I have spent with Nate and his family and Hillary and hers.

I would like to thank Dr. Allyson O’Donnell and members of the O’Donnell lab. It has been a privilege to work beside each of you over the last six years. Thank you Ally for supporting me through this journey and relaying your excitement for science to the members of your lab. To my lab family over the years, Ceara McAtee, Wes Bowman, Dr. Annette Chiang, Nejla Ozbaki Yagan, Mitch Lesko, Justina Warnick, Katie Oppenheimer, Eric Jordahl, Sydney Davis, and many others. Thank you to my cohort at Duquesne and the University of Pittsburgh, especially Charlotte,
Collin, Brandon, and Sarah. Thank you for making the last few years so memorable, I truly could not imagine this experience without each of you. Additionally, thank you to the Brodsky lab members for welcoming us to the University of Pittsburgh. Thank you to my thesis committee members, Dr. Jeff Brodsky, Dr. Jon Boyle, Dr. Anne Carlson, and Dr. Arohan Subramanya for your support during my time at the University of Pittsburgh. Your guidance and support in the form of encouragement and letters of reference have played a key role during my experience. Thank you to my undergraduate mentor, Dr. Regina Lamendella, who further inspired me to attend graduate school and has always been a source of guidance and encouragement.

Last of all I would like to extend my gratitude to all the others that have helped me along this journey. To my friends who have spent hours on the phone with me and those that I spent the last six years playing basketball, softball, bocce ball, and just enjoying life with, I will always appreciate the outlet you have given me to be someone outside of the lab. I have been so lucky to be surrounded by such amazing people.
1.0 Introduction

In this section, I discuss a class of proteins known as the inwardly rectifier potassium channels or Kir Channels. These channels play a central role in maintaining resting membrane potential and regulating action potentials in excitable cells (Hibino et al., 2010). I first detail each subfamily member, and then focus on one member of the Kir channel family, Kir2.1. Specifically, I describe Kir2.1 trafficking and disease-linked trafficking mutations. I review the studies that use yeast as a model organism that have helped define the Kir2.1 trafficking pathways. Next, I provide background on a family of protein trafficking adaptors, the α-arrestins, that we have shown play a role in Kir2.1 trafficking. I discuss and give examples of 1) how α-arrestins aid in the endocytosis of membrane proteins, and 2) their role in intracellular sorting of membrane proteins. Portions of this chapter have been adapted from a recently published review in *Frontiers in Cell and Developmental Biology* (Hager et al., 2021) and a review in preparation for *Microbiology and Molecular Biology Reviews*.

1.1 Kir2.1 function and trafficking

In order to ensure optimal cell and organismal health, potassium homeostasis must be tightly regulated. The inability to adequately control potassium balance results in disease, such as cardiac arrhythmias or development disorders. One way cells maintain optimal potassium (K⁺) levels is through the actions of a family of inwardly rectifying potassium (Kir) channels (Hibino et al., 2010). In addition to these channels, it is important to note that other channels like the Na⁺/K⁺
pump also play a critical role in regulating K⁺ balance. Kir proteins are encoded by the KCNJ gene family. Kir channels are comprised of a tetramer of Kir subunits, each of which contains two transmembrane domains, cytoplasmic N- and C-termini, and a re-entrant loop that forms an ion selectivity pore for K⁺ to pass through (Hibino et al., 2010). These channels play a role in several critical biological processes such as muscle, nerve, and immune function (de Boer et al., 2010).

Although each member of the Kir family plays a significant role in potassium homeostasis, Kir2.1 is perhaps one of the best studied members of this channel family due to its critical roles in regulating heart rhythm, muscle contraction and bone development (Figure 1). Since the Kir channel family is involved in a diverse set of biological processes, it is no surprise that mutations in Kir channels result in a multitude of diseases, ranging from cardiomyopathies to neurological and metabolic disorders. Furthermore, aberrant protein trafficking can lead to too much, too little, or malfunctioning channels at the cell surface, causing disease. Malfunctional Kir channels give rise to developmental defects such as underdeveloped skeletal systems and craniofacial and cerebellar abnormalities (Grandy et al., 2007; Harrell et al., 2007; Karschin and Karschin, 1997; Ruan et al., 2008). More specifically and relevant to my studies, mutations in Kir2.1 result in Andersen-Tawil Syndrome (ATS). ATS is characterized by periodic paralysis, cardiac arrythmia, and developmental defects (Perez-Riera et al., 2021; Plaster et al., 2001). In order to create appropriate treatments for this Kir2.1-linked disorders, we must first understand Kir2.1 channel regulation. The following chapter first gives a broad overview of the roles of Kir channels and subfamilies, then focuses on family member Kir2.1. I begin this section by giving a broad overview of the Kir channels as a whole and I discuss each subfamily within its four distinct functional groups, which include classical Kir channels (Kir2.x), G-protein-gated Kir channels (Kir3.x), ATP-sensitive K channels (Kir6.x) and K⁺ transport channels (Kir1.x). Then I describe how Kir
channels maintain K\(^+\) balance with an explicit focus on the current understanding of Kir2.1 channel trafficking highlighting the noteworthy roles Kir2.1 plays in development and disease.

1.1.1 KCNJ Family

Potassium homeostasis is critical for optimal cell health and thus cellular potassium (K\(^+\)) balance is tightly regulated within organisms. Specifically, K\(^+\) concentration regulation is essential for the normal functioning of nerve and muscle cells due to the affect it has on membrane potential (Bia and DeFronzo, 1981; Youn and McDonough, 2009). Since diet continuously influences K\(^+\) homeostasis within an organism, keeping circulating K\(^+\) levels constant is complex and critical (Youn and McDonough, 2009). Several ion channels are responsible for maintaining K\(^+\) balance, including a family of inwardly rectifying potassium (Kir) channels. This family of Kir channels is encoded by 16 KCNJ genes and together they contribute to a diverse set of cellular processes. Each channel has the same basic composition; four subunits that have been found to be predominately homo-tetramers, but occasionally form hetero-tetramers (de Boer et al., 2010; Dhamoon et al., 2004; Preisig-Muller et al., 2002; Schram et al., 2002; Zobel et al., 2003). Each subunit has two transmembrane domains, cytoplasmic N- and C-termini, and a re-entrant loop that establishes the pore-lining selectivity filter that permits the passage of K\(^+\) through the channel (Figure 2A) (de Boer et al., 2010; Hibino et al., 2010). Although Kir channels are structurally similar, they have key amino acid differences that give rise to drastically different K\(^+\) transporting abilities. For example, some channels are considered “strong” (Kir2 and Kir3), “intermediate” (Kir4), or “weak” (Kir1 and Kir6) potassium rectifiers. This is based on their ability to import potassium into the cell. In turn, this allows Kir channels to be expressed and influence K\(^+\) homeostasis in a diverse set of cell types, including cardiomyocytes, neurons, blood cells, osteoclasts, endothelial cells,
glial cells, epithelial cells, and oocytes (Hibino et al., 2010). My work primarily focused on Kir2.1, a member of the Kir2 subfamily (Kir2.1-4 and Kir2.6), historically known as the ‘classical’ Kir channels (Hibino et al., 2010).

**Figure 1. Kir Family localization**


The Kir2 subfamily (referred to hereafter as Kir2.x), which includes Kir2.1 (*KCNJ2*), Kir2.2 (*KCNJ12*), Kir2.3 (*KCNJ4*), Kir2.4 (*KCNJ14*) and Kir2.6 (*KCNJ18*) make up the “classical” Kir channels (Table 1). These channels are constitutively active and have a strong inward K$^+$ rectification. These channels are essential in establishing a stable, negative resting membrane potential in a variety of excitable cell types such as cardiomyocytes and skeletal
muscles (de Boer et al., 2010). The strong inward rectification, like we see with Kir2.1, results from a voltage dependent block of the channel by intracellular polyamines and Mg\textsuperscript{2+} (de Boer et al., 2010). Specifically, positively charged polyamines and Mg\textsuperscript{2+} enter the permeation pathway to stop K\textsuperscript{+} conductance when the membrane potential is more positive than the equilibrium potential for K\textsuperscript{+}, which is responsible for both sterically and electrostatically blocking K\textsuperscript{+} efflux (de Boer et al., 2010). This block then aids in delaying new action potential firing and ultimately ensures the appropriate QT interval is reached. Unsurprisingly, since these channels are involved in regulating homeostasis, defective channels have disastrous results. This is exemplified when we look at the well-studied, hypomorphic Δ314-315 Kir2.1 mutation (Figure 2A; Table 1), which impairs Kir2.1 trafficking to the plasma membrane (PM) thus resulting in severe Andersen-Tawil syndrome (ATS) (see sections 1.1.2.1-1.1.2.2). ATS is characterized by periodic paralysis, repolarization changes in electrocardiograms, and developmental abnormalities that consist of dysmorphic features, wide-set eyes, low-set ears, broad forehead, small jaw and head, cleft palate, and curved, fused and/or shortened digits (Perez-Riera et al., 2021; Plaster et al., 2001). At the opposite end of the spectrum, hypermorphic mutations, such as E299V, M301K and D172N, cause excess K\textsuperscript{+} flux, resulting in short QT syndrome as well as an increased risk of sudden cardiac death (Ambrosini et al., 2014; Li et al., 2004; Priori et al., 2005). Because such severe implications arise with defective Kir2.1 function, considerable research effort has revealed key mechanistic details of the channel and its regulation (see section 1.1.2.2).

Although Kir2 channels are historically recognized to have a strong inward rectification, it should be highlighted that this degree of K\textsuperscript{+} rectification by Kir2.x channels is determined by the charge of key residues in the second transmembrane helix (TM2) and that this degree of rectification varies across subfamily members. This complicates the historical accuracy of the
description of “classical” Kir channels. For example, there is a negatively charged residue (D₁₇₂) found in Kir2.1 that gives rise to its strong inward K⁺-rectification. This is caused by the increased affinity of aspartic acid (D) to intracellular polyamines/Mg²⁺, whereas an uncharged residue like asparagine (N) (N₁₇₁ in Kir1.1 or D₁₇₂N mutant of Kir2.1), gives rise to a weak inward K⁺ rectification because its affinity is diminished and polyamines/Mg²⁺ no longer block potassium from flowing through the channel (Lu and MacKinnon, 1994; Stanfield et al., 1994; Wible et al., 1994; Yang et al., 1995).

Kir2.x family members have been found to be present in a wide variety of cell types including brain, eye, heart, smooth and skeletal muscle, and kidney. Interestingly, select Kir members that play a critical role in proper development are expressed during embryonic development in the heart (Kir2.1-2.4), brain (Kir2.1-Kir2.3), limbs (Kir2.1), ear (Kir2.1), epithelia (Kir2.2), metanephros (Kir2.2), and peripheral nervous system (Kir2.2) (Grandy et al., 2007; Harrell et al., 2007; Karschin and Karschin, 1997; Ruan et al., 2008).

In recent years we have gained considerable knowledge on Kir channel function, yet there is still more to be understood about Kir physiology. Most notably and of interest to us, is that while great strides are being made to understand the trafficking of Kir2.x channels, there are still unknown molecular players that promote or inhibit Kir channels’ ability to traffic to and from the cell surface. In the following pages, I provide a synopsis of Kir2.x family members, focusing on Kir2.1 trafficking pathways. Moreover, I emphasize that disruption of Kir2.1 regulatory pathways results in a disease state and the ways in which we are able identify the trafficking machinery involved. But first, I briefly give background on the other members that make up the Kir family and their specific trafficking characteristics (Figure 1).
1.1.1.1 G-Protein-Gated Kir Channels (GIRK1-4/Kir3.1-Kir3.4)

G protein-gated inwardly rectifying potassium (GIRK/Kir3) are potassium channels with a strong inward current. They are targeted by and thus stimulated by the βγ subunits of G-protein coupled receptors (Hibino et al., 2010). As a result, Kir3 channels remain closed until activated by the βγ subunits which creates a conformational change (Jin et al., 2002). The Kir3/GIRK subfamily consists of four members: Kir3.1/GIRK1 (KCNJ3), Kir3.2/GIRK2 (KCNJ6), Kir3.3/GIRK3 (KCNJ9), and Kir3.4/GIRK4 (KCNJ5). They can form either hetero- or homo-tetramers with various combinations of Kir3 subunits. These combinations allow them to localize to and influence a broad range of tissues and physiological responses (Ma et al., 2002). For example, GIRKs are found in the central nervous system where they contribute to cerebral currents as well as the heart where they play a role in parasympathetic slowing of the heart rate (Luscher and Slesinger, 2010; Touhara et al., 2016). Malfunction (loss of function, gain of function, or reduced function) of the GIRK channels result in diseases like epilepsy, addiction, Down’s syndrome, ataxia, and Parkinson’s disease (Luscher and Slesinger, 2010). Interestingly, due to their role in addiction many studies involving cocaine and methamphetamine have elucidated the relationships between select GPCRs and the Kir3 subfamily (Luscher and Slesinger, 2010).

GIRK channels have a variety of complex trafficking patterns that allow them to partake in a diverse set of roles. Like “Classical” Kirs of the Kir2.1 family (see below), the Kir3.x channels also require an ER export signal to traffic out of this compartment and eventually to reach the PM. Interestingly, several Kir3.x family members, namely Kir3.1 and Kir3.3, lack these ER export signals (Ma et al., 2002) and so are retained in the ER when forming homo-tetramers. In contrast, Kir3.2a and Kir3.4 homo-tetramers successfully traffic to the PM as they contain the needed ER export motifs (NQDMEIGV or DQDVESPV in or Kir3.2 or 3.4, respectively) as well as a post
Golgi surface-promoting motif located in the distal C-terminal region (Ma et al., 2002). Therefore, in order for Kir3.1 or Kir3.3 to be trafficked to the PM they must form hetero-tetramers with either Kir3.2 or Kir3.4. Furthermore, Kir3.2 encompasses an endocytosis signal in amino acids 7-14 in the N terminus (TESMTNVL) (Ma et al., 2002). When this sequence is intact endogenous Kir3.2 primarily localizes to the cytoplasm in hippocampal neurons and to intracellular vesicles in COS7 cells, yet mutation of this sequence (VL) leads to increased PM localization and decreased intracellular localization (Chung et al., 2009; Ma et al., 2002). Interestingly, when the glutamate receptor and ion channel, N-methyl-D-aspartate receptor (NMDAR), is activated, Kir3.1 and Kir3.2 surface expression is increased. This increase in surface expression is dependent on the protein phosphatase-1-mediated dephosphorylation of the Ser-9 residue on Kir3.2 which precedes the internalization motif (Val-13/Leu-14) (Chung et al., 2009). Additionally, unlike the other subfamily members, Kir3.3 contains a lysosomal targeting sequence (YWSI) that negatively impacts its PM localization. Thus, when hetero-tetramers encompass Kir3.3, they show less PM localization (Ma et al., 2002). Furthermore, Kir3 channels have been shown to traffic with their respective GPCR and this regulation is controlled via phosphorylation of either Kir3 (Kir3.2 Ser-9) or the GPCR (GABA_B Ser-783) (Clancy et al., 2007; Hearing et al., 2013; Padgett et al., 2012). Interestingly, it has been suggested that there is a sex-dependent trafficking difference of GABA_B-GIRK regulation via phosphorylation (Marron Fernandez de Velasco et al., 2015). Due to the close relationship with GPCRs there is much to still be learned about the trafficking of G protein-gated inwardly rectifying potassium as they would be prime candidates for therapeutic targets.
1.1.1.2 $\text{K}_\text{ATP}$ Channels (Kir6.1 and Kir6.2)

The $\text{K}_\text{ATP}$ channels include two members: Kir6.1 ($\text{KCNJ8}$) and Kir6.2 ($\text{KCNJ11}$). Unlike “Classical” Kir channels, ATP-sensitive potassium channels have a weak inward rectification that is inhibited by ATP and activated by nucleotide diphosphates (NDPs) like Mg-ADP (Tung and Kurachi, 1991). There are two components needed for $\text{K}_\text{ATP}$ channels; a Kir6.x (Kir6.1 or Kir6.2) subunit and the protein sulfonylurea receptor or SUR (SUR1 or SUR2), which is a member of the ATP-binding cassette (ABC) transporter superfamily (Hibino et al., 2010). The location and conductance strength of the $\text{K}_\text{ATP}$ channels differ according to the subunit makeup, but they can be found in cardiac myocytes, pancreatic $\beta$-cells, skeletal muscle, smooth muscle, brain, pituitary, kidney, and in the mitochondria (Hibino et al., 2010). In the pancreas, $\text{K}_\text{ATP}$ channels close in response to high blood glucose levels to depolarize $\beta$-cells and allow for insulin secretion. Specifically, when ATP is around, $\text{K}_\text{ATP}$ channels close (Sivaprasadara et al., 2007). $\text{K}_\text{ATP}$-associated diseases are linked to aberrant insulin secretion like congenital hyperinsulinism (CHI), and neonatal diabetes mellitus (NDM) (Sivaprasadara et al., 2007).

Proper trafficking of Kir6.2 is essential as mutations that interfere with its PM localization result in CHI (Congenital hyperinsulinism). CHI causes abnormally high levels of insulin and results in episodes of low blood sugar (Sivaprasadara et al., 2007). Importantly, Kir6.2 must bind with SUR1 in order to traffic to the PM as a recognition sequence for COPI proteins (amino acids RKR) on Kir6.2 ensures that unbound Kir6.2 does not traffic to the PM. Specifically, this recognition sequence causes the rerouting of unbound subunits to the ER (Yuan et al., 2003). Additionally, when subunits are assembled correctly, they bind with 14-3-3 proteins (Heusser et al., 2006). This promotes cell surface expression because the 14-3-3 proteins block the COPI recognition sequence (Heusser et al., 2006). Furthermore, the mutation $\text{E282K}$ disrupts the ER
exit signal and prevents Kir6.2 incorporation into COPII vesicles thus decreasing its PM localization (Sivaprasadarao et al., 2007). Once at the PM, Kir6.2 is rapidly endocytosed via a binding sequence with the clathrin adaptor complex, AP2, in the C-terminus (\text{330YSKF333}) (Mankouri et al., 2006). Mutations in this region (\text{Y330C} and \text{F333I}) cause an increase in Kir6.2 at the PM resulting in permanent neonatal diabetes mellitus (Mankouri et al., 2006).

1.1.1.3 K$^+$ Transport Channels (Kir1.1, Kir4.x, Kir5.1, Kir7.1)

The K$^+$ transport family consists of four subfamilies that are involved in a variety of cellular processes and have a range of K$^+$ conductance. The K$^+$ Transport Channel Kir1.1 (also known as the “rat outer medullary K$^+$ channel (ROMK)) was the first Kir channel to be identified (Hibino et al., 2010). It has a very weak potassium rectification due to the uncharged residue in the TM2 helix (see above) and contributes to salt and water homeostasis in the kidneys. Kir1.1 has three splice variants (Kir1.1a-c) that are functionally similar, but each expressed differentially along the nephron (Boim et al., 1995; Shuck et al., 1997). Much of what is known about the its function has been discovered by studying the Kir1.1-linked disease Bartter’s syndrome (Simon et al., 1996). Bartter’s syndrome is characterized by the presence of hypokalaemic metabolic alkalosis (potassium deficiency) and hypercaliuria (excess calcium in urine) thus resulting in salt wasting and low blood pressure (Simon et al., 1996).

Unlike other Kir family members, Kir1.1 possesses an ER retention signal (amino acids RXR) in the COOH terminus, and is dependent on posttranslational modification to traffic to the PM (Hibino et al., 2010). PM localization is dependent on posttranslational modifications like phosphorylation by PKA or SGK-1 (S44) (Hebert et al., 2005; Yoo et al., 2003). Phosphorylation at S44 suppresses the ER retention signal allowing properly folded and mature Kir1.1 to advance
to the PM (O’Connell et al., 2005). Further work showed that increased PM localization of Kir1.1 regulation by SGK-1 was dependent on a scaffolding protein, Na\(^+\)-H\(^+\)-exchanger regulatory factor-2 (NHERF-2) (Yun et al., 2002). Additionally, Kir1.1 PM expression is suppressed by select serine-threonine kinases –WINK1, WINK2, and WINK4– via different mechanisms (Kahle et al., 2003; Leng et al., 2006; Wade et al., 2006). Once ROMK is at the PM, it is internalized via a clathrin-dependent mechanism that relies on an NPXY motif (Zeng et al., 2002). In fact, WINK4 inhibits Kir1.1 activity by inducing its removal from the PM via clathrin-dependent endocytosis (Kahle et al., 2003). Another posttranslational modification – mono-ubiquitination – of Kir1.1 also regulates its endocytosis (Lin et al., 2005). Furthermore, Mackie et al. identified trafficking factors involved in the post-endocytic pathway that negatively impact Kir1.1 channels at the PM. Specifically, the endosomal complexes required for trafficking (ESCRT) and the class-C core vacuole/endosome tethering (CORVET) complex reduced Kir1.1 function at the PM (Mackie et al., 2018).

The K\(^+\) transport channels Kir4.1-2 and Kir5.1, which can form either homo- or hetero-tetramers have been found in a variety of cell types including the stomach, ear, glial cells, kidney, and retina (Hibino et al., 2010). Due to their broad localization patterns, deletion or mutation of these channels result in an assortment of diseases. For example, mutations in Kir4.1 results in EAST/SeSAME syndrome, which is characterized by epilepsy, ataxia, seizures, sensorineural deafness, ataxia, intellectual disability, and electrolyte imbalance. Kir4.1 contributes to potassium homeostasis in the retina by maintaining the resting membrane potential. Inability of Kir4.1 function causes an increased glutamate efflux and excessive potassium ions in Müller cells increases osmotic pressure and leads to swelling and eventual cell autophagy and apoptosis (Djukic et al., 2007; Reichenbach and Bringmann, 2013). This suggests that Kir4.1 plays a role in diabetic
retinopathy (Li et al., 2021). Furthermore, the relationship between Kir4.1 and glutamate receptors is important as abnormal activity of glutamate receptors results in neurological diseases such as Alzheimer’s disease, amyotrophic lateral sclerosis (ALS) and Huntington’s disease (Lin et al., 1998; Scott et al., 2011).

EAST/SeSAME also results from the mutation A167V, which inhibits the ability of Kir4.1/Kir5.1 channels to traffic to the surface of tubular cells causing renal salt wasting (Tanemoto et al., 2014). On the other hand, an increased localization of Kir4.1 channels at the PM caused by a mutation (R18Q) causes a gain-of-function effect that is found in children with autism and epilepsy (Sicca et al., 2016).

Kir7.1 (KCNJ13) functions as a homo-tetramer and has a low channel conductance that is the result of methionine in the pore complex (M125), which is an arginine in other Kir channels (Hibino et al., 2010). Kir7.1 can be found in the retinal pigmented epithelia (RPE) and epithelial cells in the choroid plexus (Hibino et al., 2010; Krapivinsky et al., 1998). Additionally, in rats it was also found in the distal convoluted tubule, cortical collecting duct, connecting tubule, thick ascending limb and the outer medullary collecting duct (Ookata et al., 2000). Defects in Kir7.1 cause Snowflake vitreoretinal degeneration (SVD) and Leber congenital amaurosis (LCA). SVD is an autosomal-dominant disease that is characterized by fibrillar degeneration of the vitreous humour, early-onset cataract, minute crystalline deposits, and retinal detachment (Hirose et al., 1974; Robertson et al., 1982) and LCA is characterized by serve visual impairment from birth (den Hollander et al., 2008). Trafficking of Kir7.1 is understudied, however, mutations in the COOH terminus (R166X and L241P) lead to loss of the C-terminus and failure to traffic to the PM, but the impact of these mutations still needs to be completely characterized (Sepulveda et al., 2015).
1.1.2 Regulation of Kir2.x by protein trafficking and its role in disease

1.1.2.1 Mapping the Kir2.1 trafficking itinerary: machinery and motifs

Several trafficking factors have been identified in the Kir2 family. For example, all Kir members possess an ER export sequence located in their cytoplasmic C-termini (consensus sequence $\text{FXYENEV}^{374}\text{FCYENEV}^{380}$ for Kir2.1), which is necessary for Kir2.x ER exit (Ma et al., 2001; Stockklausner et al., 2001) (Figure 2A-B). Additionally, the cytoplasmic portion of the C-terminal tail in Kir2.1 was found to interact with the GRIP (golgin-97, RanBP2alpha, Imh1p and p230/golgin-245) domain in Golgin-97. Golgin-97 is a member of the Golgin family of membrane and cytoskeleton tethers that are responsible for capture and transport of vesicle fusion to Golgi compartments. This interaction via the GRIP domain of Golgin-97 helps ensure that Kir2.1-containing vesicles are able to reach their correct location in the trans-Golgi network (TGN) (Taneja et al., 2018) (Figure 2B). Experiments where Taneja et al. knocked down Golgin-97 reported significant reduction of Kir2.1 localization at the PM. Furthermore, interaction with Golgin-97, they hypothesized, supports the incorporation of Kir2.1 into AP-1 associated vesicles (Taneja et al., 2018).

Once at the TGN, Kir2.1 directly interacts with adaptin protein complex 1 (AP-1). The AP-1 complex is a cargo selective adaptor for Golgi- and endosome-derived, clathrin-coated transport vesicles (Bonifacino and Traub, 2003; Ma et al., 2011) (Figure 2B). AP-1 is a key player in Kir2.1 trafficking to the PM. Disruption of this interaction via the mutant, Kir2.1-$\Delta$314-315, leads to failure of Kir2.1 to exit the Golgi and results in a severe ATS phenotype (Ma et al., 2011). Interestingly, direct interaction between Kir2.1 and AP-1 is dependent on both the N- and C-terminal residues of Kir2.1. Initial studies identified basic residues in the N-terminus ($R^{44}$ and $R^{46}$ in Kir2.1) that when mutated to alanine were able to stop Golgi export (Figure 1A)
These N-terminal residues were found to form an interface with residues found in the C-terminus (Y315, E319, I320 and W322). This formation is what allows for AP-1 to bind and for Kir2.1 to be exported. This two-fold interaction motif is structurally distinct from the conventional AP-1 motifs seen previously (reviewed in (Traub and Bonifacino, 2013)), yet interestingly it seems to be conserved in many of the Kir channel proteins (Li et al., 2016). It should be noted that earlier studies did identify a more ‘classical’ AP-1 binding sequence in the C-terminus of Kir2.1 with the consensus YxxΦ, where x is any amino acid and Φ is a bulky hydrophobic amino acid (corresponds to amino acids 242YIPL245 in Kir2.1; Figure 2A), as important for Golgi-to-PM trafficking (Hofherr et al., 2005). This YxxΦ is actually present in all Kir2.x family members, which is puzzling since subfamily members do not all traffic to the PM at the same frequency. Hofherr et al. suggests that flanking residues contribute to the difference and ultimately control signal recognition (Hofherr et al., 2005). Additionally, more recent mutational analysis further calls into question if this patch plays a role in Kir2.x family member’s sorting to the surface (Li et al., 2016). Kir2.1 binds to the γ-aminobutyric acid type A (GABA_A) receptor interacting factor-1 (GRIF-1; aka trafficking kinesin protein 2 [TRAK2]) to help facilitate its transition from the Golgi to the PM. GRIF-1 is responsible for linking kinesin heavy chains to specific cargo, like Kir2.1, and is suspected to play a role in anterograde trafficking of vesicles and organelles (Smith et al., 2006). Girshin et al. were able to identify the interaction between GRIF-1 and Kir2.1 by using yeast 2-hybrid (Grishin et al., 2006). Specifically, they found that the interacting region resides within the N-terminus of GRIF-1 and amino acids 348-396 in the C-terminus of Kir2.1 (Figure 2A). Additional experiments showed that the GRIF1-Kir2.1 interaction promotes Kir2.1 localization to the PM (Figure 2B) (Grishin et al., 2006).
Kir2.1 must reach the PM to allow extracellular K\(^+\) into the cell. Once at the PM, several factors affect its activity, localization, and cell surface dwell time. For example, Kir2.x channels are known to bind with lipids, including phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)), which initiates channel opening (Hansen et al., 2011). X-ray crystallography studies using subfamily member Kir2.2 have been able to shed light on the relationship between the Kir2 family and PIP\(_2\). Specifically, PIP\(_2\) binds Kir2.1 between its transmembrane domain and cytoplasmic domain and once bound generates a large conformational change resulting in K\(^+\) passage through the pore (Hansen et al., 2011; Huang et al., 1998). The conformation change initiated by PIP\(_2\) allows for proper regulation by Mg\(^{2+}\). Consequently, if PIP\(_2\) binding is disrupted it leads to irreversible inhibition of Kir2.1 by Mg\(^{2+}\) (Du et al., 2004). The inability of PIP\(_2\) to bind residues of Kir2.1 is associated with ATS (see section 1.1.2.2 below).

Kir2.1 localization at the PM is dependent on PM makeup. For example, Kir2.1 partitions to cholesterol- and flotillin-rich membrane fractions (Ambrosini et al., 2014; Tikku et al., 2007). In support of this, cholesterol binding sites were mapped in Kir2.1 in its TMD \(\alpha\)-helices via computational and mutagenesis strategies (residues L69, A70, V77, L85, V93, S95, I166, V167, I175, M183, Y68, C76, I79, F159, and S165) (Figure 2A) (Rosenhouse-Dantsker et al., 2011; Rosenhouse-Dantsker et al., 2013). Not only does cholesterol have a hand in partitioning of Kir2.1, but when there is an increase in membrane cholesterol there is also an increased probability of channel closure. Thus cholesterol regulates Kir activity via cholesterol-responsive sites which are different from the cholesterol binding sites mentioned above (Romanenko et al., 2004; Rosenhouse-Dantsker et al., 2011). Furthermore, cholesterol/flotillin-rich membranes are often associated with caveolins. Caveolins are proteins (Cav1, Cav2, and Cav3) that make up caveolae, which operates as macromolecular vesicular transporters and can be thought of as PM
lipid rafts (Cohen et al., 2004). Kir2.1 interacts with each of the caveolins (Ambrosini et al., 2014; Han et al., 2014; Ikezu et al., 1998; Tikku et al., 2007; Vaidyanathan et al., 2018). While we would expect to see a role for caveolins in the trafficking, specifically endocytosis, of Kir2.x proteins this remains largely unexplored. Yet, we do see them as negative regulators due to suppression of Kir2.x current. Specifically, Cav1 and Cav3 are negative regulators of Kir2.1 via dampening of Kir2.x current. Additionally, Cav3 mutations alone can cause long-QT syndrome and are associated with decreased Kir2.1 current (Han et al., 2014; Vaidyanathan et al., 2018). Cav3 binds Kir2.1 between amino acids 81-88 in the N-terminus of this protein, via a canonical \( \Omega \times \Omega \times \times \times \Omega \) Cav3-binding motif (W is any aromatic amino acid; \(^{81}WRWMLVIF^{88}\) for Kir2.1 and shown as CBS in Figure 1A) (Vaidyanathan et al., 2018).

At the PM Kir2.x channels form macromolecular complexes that help stabilize Kirs at the cell surface. This is highlighted by the fact that when at the PM Kir2.x interact with many postsynaptic density, discs large, and zonaula occludens (PDZ)-domain containing proteins, via a class I PDZ domain-recognition sequence \((^{440}SEI^{442})\) for Kir2.1) in their C-termini that many Kir channels possess (Cohen et al., 1996; Songyang et al., 1997). Some of the PDZ domain-containing proteins that Kir2.x interact with include the MAGUK (membrane-associated guanylate kinase) family, including SAP97, PSD-95, Chapsyn 110, and CASK. These MAGUK family of proteins serve as molecular scaffolding proteins that can regulate signaling and the trafficking of many receptors and ion channels (Cohen et al., 1996; Leonoudakis et al., 2004a; Leonoudakis et al., 2004b; Leonoudakis et al., 2001; Sheng and Sala, 2001). Kir2.x interacts with additional scaffolding and structural proteins in the brain that play a role in PM localization, such as SAP97, CASK, Veli, Mint and actin-binding LIM proteins (Leonoudakis et al., 2004b). To find more
information on how Kir2.1 functions within macromolecular complexes see Willis et al. (Willis et al., 2015).

Finally, in addition to those factors discussed above, other regulators are thought to play a role in Kir2.1 trafficking and localization to the PM. For example, recent work has shed light on the role focal adhesions (FAs) play in Kir2.1 PM localization. Confocal microscopy identified Kir2.1 accumulating in PM subdomains proximal to FAs. This study went on to show that the larger the FA area the higher the amplitude of Kir2.1 current, suggesting that they promote Kir2.1 surface accumulation whereas when Kir2.1 is in areas distal to FAs, it appears to be more thoroughly endocytosed (Sengupta et al., 2019). This trend is thought to be the outcome of local inhibition of dynamin-dependent endocytosis at FA sites, thus permitting localized Kir2.1 accumulation (Sengupta et al., 2019). Filamin-A, a protein that crosslinks actin filaments, interacts with Kir2.1 and plays a role in its cell-surface localization (Sampson et al., 2003). Using a yeast two-hybrid screen Sampson et al. identified filamin-A as a binding partner of Kir2.1 (amino acids 307-326). Interestingly, the common Δ314-315 mutant did not disrupt this binding. Moreover, when filamin is depleted from cells there is less Kir2.1 surface abundance and a reduction in K+ currents suggesting that filamin is important for insertion and/or retention of Kir2.1 at the cell surface (Sampson et al., 2003). Thus, a complex orchestration of molecular factors regulates Kir2.1, and other Kir2.x family members, trafficking and partitioning at the cell surface to promote optimal function for these critical K+ channels.

Kir2.1 surface abundance must be controlled by regulated endocytosis and subsequent lysosomal degradation, as has been described for many channels and transporters (Estadella et al., 2020). Internalization from the PM can occur via clathrin-mediated endocytosis or clathrin-independent endocytosis mechanisms (CME and CIE, respectively) (von Zastrow and Sorkin,
Surprisingly, there are currently very few descriptions of Kir2.1 endocytic regulation whereas for other Kir family members, including Kir2.3, Kir6.2 and Kir1.1 (ROMK) there is clear evidence for clathrin-mediated endocytic turnover (Mankouri et al., 2006; Mason et al., 2008; Ortega et al., 2012; Zeng et al., 2002). Interestingly, Kir2.3, which is >60% identical to Kir2.1, has a non-canonical adaptin-protein complex 2 (AP-2) binding site with a recognition motif of \(\Phi\Phi\Phi\Phi\) spanning residues \(412_{\text{IRML}416}\) (Ortega et al., 2012). Because AP-2 is an adaptor that recruits clathrin to endocytic sites, this suggests that the Kir2.x family uses CME. However, despite the >60% conservation between Kir2.1 and Kir2.3 the AP-2 binding site is not conserved in Kir2.1. Additionally, Kir6.2 has a canonical AP-2 binding site \(330_{\text{YSKF}333}\) which is consistent with a \(Yxx\Phi\) AP-2 recognition motif) (Mankouri et al., 2006). This motif is nearly perfectly conserved in Kir2.1 \(341_{\text{YSRF}344}\), but its ability to bind AP-2 has not been tested and it should be noted that this \(Yxx\Phi\) motif is distinct from the motif found between amino acids 242-245 that was initially considered as important for Golgi exit as stated previously.

An early study of Kir2.1 in Xenopus oocyte suggested that it is regulated by CME. Specifically, it was found that caging of \(Y242\) of Kir2.1 resulted in increased Kir2.1 PM localization, yet with uncaging, endocytosis of Kir2.1 was increased suggesting that phosphorylation of Kir2.1 at this residue might signal for endocytosis (Tong et al., 2001). Also, a dominant-negative mutant in dynamin prevented the \(Y242\)-induced internalization of Kir2.1, again suggesting CME trafficking of Kir2.1. It is important to note that both CME and CIE are able to engage dynamin, a small GTPase involved in membrane scission events, to release vesicles from the membrane (Mayor et al., 2014; Sandvig et al., 2018). Therefore, the demonstration that dynamin is involved does not truly define a role for CME vs CIE in Kir2.1 regulation.
Several recent studies have looked further at the role of the Rho-family GTPases (including Rho, Rac and Cdc42), which have broad cellular functions ranging from regulating protein trafficking, cytoskeletal dynamics, and cell-cell adhesion, in controlling Kir2.1 (Boyer et al., 2009; Hodge and Ridley, 2016; Mayor et al., 2014). Pharmacological inhibition or dominant negative mutations that impede the Rho, Rac and Cdc42 GTPases have been used to demonstrate that impairment of Rac1 increases Kir2.1 at the cell surface, as shown through increased conductance and localization via microscopy (Boyer et al., 2009). Interestingly, when using a dynamin mutant that inhibits vesicle scission, the impact of Rac1 is lost. This suggests the role of Rac1 is coupled with the endocytosis of Kir2.1. Of further note, Rac1, and other Rho-family GTPases can contribute to clathrin-independent, dynamin-dependent endocytic pathways and perhaps this is how Rac1 is able to control Kir2.1. (Grassart et al., 2008). Surprisingly, other members of the Kir2.x family were not found to be regulated in the same way as Kir2.1 as the Rac1 mutants did not alter their distribution. Taken together, while Kir2.1 internalization from the cell surface is regulated, it remains unclear which endocytic pathway and sorting factors control Kir2.1 endocytosis. It is tempting to speculate that a clathrin-independent route may be at play for Kir2.1 given its regulation by the Rac1 GTPase and its propensity to cluster cholesterol-rich membrane domains as well as interact with the caveolins, but this has yet to be fully assessed.

While the endocytic route for Kir2.1 remains enigmatic, it is clear that this channel transits to the lysosome for degradation as lysosomal inhibitors, such as NH₄Cl, chloroquine, and leupeptin, that disrupt lysosomal function increase Kir2.1 protein levels (Jansen et al., 2008). Further studies in yeast (as I will describe below) identified the endosomal sorting complex required for transport (ESCRT) machinery as important for controlling Kir2.1 function at the cell surface. These yeast studies showing the involvement of ESCRTs was then confirmed by studies
in HeLa cells where knockdown of ESCRT components increased Kir2.1 abundance (Kolb et al., 2014).

1.1.2.2 Disease-linked trafficking mutations in Kir2.1

Identification and characterization of mutations causing Kir2.1 loss-of-function, including those associated with ATS, have helped advance our understanding of Kir2.x trafficking. Perhaps the best characterized and most common mutation associated with ATS is a 2 amino acid deletion mutation – Δ314-15 – in Kir2.1. This deletion blocks surface expression of Kir2.1 by disrupting an unconventional trafficking signal: two patches of residues within the cytoplasmic N-and C-termini that together in its tertiary structure create a binding site for the AP-1 complex, a clathrin adaptin complex important for post-Golgi sorting of vesicles to the PM (Figure 2A-B, Table 1) (Bonifacino and Traub, 2003; Ma et al., 2011). Confocal microscopy found channel retention in the Golgi and pulse-chase experiments demonstrated that mutant channels never made it to the PM suggesting that this deletion results in a trafficking defect. Further analysis showed that the mutant channel, unlike wildtype (WT) Kir2.1, was unable to interact with AP-1 (Ma et al., 2011; Welling, 2013). These findings suggest a model whereby Kir2.1 is incorporated into AP-1 and clathrin-coated vesicles at the TGN which are then targeted to the PM (Figure 2B). BioID (proximity-dependent Biotin Identification) proximity proteomics analyses of Kir2.1-Δ314-315 further validated the spatial distribution differences compared to WT Kir2.1. Specifically, proteins involved in intracellular trafficking and transport were enriched with Kir2.1-Δ314-315 (Park et al., 2020). Thus, it is likely that Kir2.1 interacts with AP-1 and clathrin-coated vesicles at the TGN which then allows it to transit to the cell surface (Figure 2B). Unsurprisingly, patients with the Kir2.1-Δ314-315 mutation have severe ATS phenotypes (Plaster et al., 2001).
Unlike Δ314-315, other mutations in regions needed for effective trafficking of Kir2.1 led to varying degrees of ATS phenotypes. For example, patients with the S369X mutation, which results in a premature stop codon and truncation of the C-terminus, lack the ER export motif (FCYENE) and therefore have reduced Kir2.1 trafficking to the PM and lower channel activity resulting in a severe disease phenotype (Doi et al., 2011). However, when a Kir2.1 tetramer containing both a mutated S369X subunit and a wild-type Kir2.1 as a mixed heteromer is assembled the intact ER-export signal from the wild-type Kir2.1 subunits can partially suppress the trafficking defect, allowing for improved ER-export and increased Kir2.1 function. Therefore, patients that are heterozygous for S369X display only mild ATS symptoms (Doi et al., 2011).

While some Kir2.1 mutations can be compensated for through assembling with wild-type Kir2.1 subunits, like the S369X mutation, others act as dominant-negatives. This results in impairment of the co-assembled wild-type Kir2.1 subunits. This is seen with Kir2.1-Δ314-315 assembly where wild-type Kir2.1 subunits still fail to traffic to the PM, likely due to the inability to associate with AP-1 regardless of the presence of the wild-type subunit (Bendahhou et al., 2003).

There also exist ATS-linked mutations that affect the ability of Kir2.1 to reach the PM, however, a majority of these the defects are likely linked to incorrect protein folding or inability to insert appropriately into the ER rather than a direct protein trafficking defect (Bendahhou et al., 2003). These mutations include a deletion in the first membrane span (Δ95-98) thought to interfere with Kir2.1 membrane insertion and resulting in cytoplasmic channel localization (Figure 2A, Table 1) (Bendahhou et al., 2003). Mutation of V302M, which lies at the end of one of the beta-sheets (see Figure 2A) in an area where the increased side chain length for methionine may disrupt adjacent non-covalent bonds in Kir2.1’s cytoplasmic domain, also prevents Kir2.1 PM localization and is thought to be misfolded. This mutant too localizes in the cytosol, potentially due to its
recognition and retrotranslocation from the membrane as a misfolded protein by the protein quality control machinery (Bendahhou et al., 2003). Finally, mutation C101R in the first transmembrane helix when expressed in HEK293T cells has reduced PM, suggesting that this region is essential for adequate Kir2.1 PM localization, but again this seems likely due to misfolding and/or mis-insertion into the membrane as it is seen predominately in the cytosol (Figure 2A, Table 1) (Ballester et al., 2006; Bendahhou et al., 2003).

Mutations T75M and T74A in Kir2.1 inhibit phosphatidylinositol 4,5-bisphosphate (PIP2) binding. Specifically, T74A traffics like WT, but activity is altered as a result of diminished PIP2 associations (Ballester et al., 2006). T75M results in decreased levels at the PM compared to WT. Although the trafficking of T75M is restored when co-expressed with WT Kir2.1, there still persists decreased activity likely due to defective PIP2 interaction (Tani et al., 2007). Additionally, R218W, G300V, E303K also disrupt PIP2 binding, but have not been tied to Kir2.1 mis-trafficking (Lopes et al., 2002). A mutation adjacent to the PIP2 binding site (H53), G52V, results in Golgi retention. This retention could be the result of the disruption of a nearby AP-1 binding site (residues 44-46 and 314-322) or it may act like the T75M mutant and alter the PIP2 interaction thus disrupting trafficking (Gelinas et al., 2017).

Only one gain-of-function trafficking-defective mutant has been found thus far: Kir2.1-K346T, which is linked to short QT syndrome 3 (Ambrosini et al., 2014). This mutation causes retention at the PM, diminished caveolins interactions, increased partitioning to cholesterol-poor membranes, and reduced ubiquitination of the mutant (Ambrosini et al., 2014). Interestingly, the decrease in ubiquitination levels may indicate this site is involved in ubiquitin-dependent endocytosis, but this has yet to be explored.
Figure 2. Structural mapping and trafficking model of Kir2.1.

(A) A model for the Kir2.1 monomer was generated using AlphaFold2 by Mitchell A. Lesko (accession #P63252) (Jumper et al., 2021). Interestingly, the AlphaFold2 predicted structure for Kir2.1 reveals an extended alpha-helix spanning residues 358-388 that is not observed in the crystal structures for Kir channels as this region was removed.
from the protein prior to crystallization. For simplicity a dimer of Kir2.1 subunits is shown, rather than the tetramer that would typically exist at the PM. The left subunit on the dimer denotes binding motifs for trafficking factors and the right subunit highlights disease-causing mutations associated with aberrant Kir2.1 trafficking. Left: Selectively filter (pink), Mg$^{2+}$/spermine binding site (gray and green where it overlaps with a cholesterol binding residue), Cholesterol binding residues (yellow except where they overlap with a Mg$^{2+}$/spermine binding site [light green], a Cav3 binding site [dark green], or a PIP$_2$ binding site [orange]), Cav3 binding site (CBS, bright green and one dark green where overlaps with cholesterol binding site), PIP$_2$ binding residues (red and one orange where it overlaps with a cholesterol binding site), YxxΦ motif (pink), AP-1 binding site (blue), GRIF-1 interaction region (tan), and ER export signal (purple). Right: The side chains of residues mutated in disease and linked to defective protein trafficking are shown as spheres. Text giving the specific mutation associated with each is color-coordinated. (B) Trafficking model of Kir2.1 highlighting interacting components at each stage including: Golgin-97, AP-1, GRIF-1, dynamin, Rac1, retromer, and ESCRT. Purple arrows indicate well-studied trafficking routes for Kir2.1 whereas the red arrow denotes a putative endosome-Golgi recycling pathway. MVB, multivesicular bodies; ER, endoplasmic reticulum; PM, plasma membrane.

1.1.2.3 Kir2.1 trafficking studies in *Saccharomyces cerevisiae*

Studies in *Saccharomyces cerevisiae* as a heterologous expression system for Kir2.1 have contributed meaningfully to our knowledge of Kir2.1 trafficking and degradation (Hager et al., 2018; Kolb et al., 2014). Initial studies ectopically expressed Kir2.1 in yeast strains lacking endogenous potassium Trk1 and Trk2. In this model, Kir2.1 becomes the primary mode of import for potassium into yeast cells, so when these cells are grown on potassium restrictive medium, the cells are dependent on Kir2.1; thus, growth on potassium restrictive medium serves as a proxy for Kir2.1 function at the cell surface. Taking advantage of this system, Kolb *et al.* performed a genome wide systematic screen to identify gene deletions that improve Kir2.1-dependent growth on low K$^+$ medium. From this screen, over 60% of the top candidates identified were trafficking factors. These were identified as putative negative regulators of Kir2.1 activity at the cell surface.
and include retromer (retrieval of vacuolar-targeted proteins), AP-1 (clathrin adaptor protein complex), and ESCRT as each of these genes when deleted improved growth on low potassium medium (Kolb et al., 2014). From these putative regulators, components of the ER-associated degradation (ERAD) and ESCRT pathways were validated. Specifically, in the yeast system Kir2.1 is a target of ERAD that requires Cdc48, an AAA-ATPase, the ER-associated E3 ubiquitin ligases Hrd1 and Doa10, and Ssa1, a cytoplasmic Hsp70 chaperone for retro-translocation and degradation (Kolb et al., 2014). Deletion or impairment of these factors in yeast leads to stabilization of Kir2.1 protein. For the ESCRT regulation, several components of the pathway were identified in the initial yeast screen (Did2, Vps36, Vps27, Vps22, Vps2, Vps23, Vps37, Mvb12, and Vta1) and deletion of these components led to increased Kir2.1 dependent growth on low potassium medium, suggesting that these factors are needed to degrade Kir2.1. ESCRT degradation of Kir2.1 likely occurs through post-endocytic trafficking to the vacuole, which is the yeast equivalent of the lysosome. Excitingly, the dependence on ESCRT for lysosomal degradation of Kir2.1 was validated in HEK293T cells. This demonstrates that this yeast system where Kir2.1 is ectopically expressed is able to define relevant regulators of its trafficking (Kolb et al., 2014).

In subsequent studies using this yeast model, the α-arrestins, a class of selective protein trafficking adaptors that will be discussed further below (Becuwe et al., 2012; Lin et al., 2008; Nikko and Pelham, 2009; Nikko et al., 2008; O'Donnell and Schmidt, 2019; Patwari and Lee, 2012), were identified as key regulators of Kir2.1 trafficking (Hager et al., 2018). The α-arrestins selectively bind to membrane proteins and recruit a ubiquitin ligase that in turn ubiquitinates membrane proteins, which alters its protein trafficking. Ubiquitination often serves as a signal for endocytosis or intracellular sorting for membrane cargo proteins (Becuwe et al., 2012; Lin et al., 2008; Nikko and Pelham, 2009; Nikko et al., 2008; O'Donnell et al., 2010) and the α-arrestins are
a key facet of this protein trafficking pathway. In yeast, α-arrestins bind to the ubiquitin ligase, Rsp5, the ortholog of which is mammalian Nedd4-2. Select yeast α-arrestins—Ldb19/Art1, Aly1/Art6, and Aly2/Art3—promote Kir2.1 trafficking to the cell surface, increasing Kir2.1 activity at the PM, and thereby raising intracellular potassium levels and improving growth on potassium restrictive medium. In addition, regulators of these α-arrestins, including Rsp5 and the protein phosphatase calcineurin, which is conserved from yeast to humans and is highly expressed in the heart and other excitatory cells where Kir2.1 function is important, were also identified in the yeast model (Hager et al., 2018). Current and future studies in the O’Donnell lab will determine if these factors also control Kir2.1 trafficking in mammalian systems. Additionally, it is important to note that studies of another Kir family member, Kir1.1 (ROMK), in a yeast model system have been influential in defining key trafficking factors for this channel using a similar yeast model system, just with Kir1.1 in place of Kir2.1 (Mackie et al., 2018). Specifically, using this yeast model system Mackie et al. conducted a synthetic genetic screen to identify nonessential genes that were involved in trafficking Kir1.1 away from the PM. Using this approach, they identified members of both the endosomal complexes required for transport (ESCRT) and the class-C core vacuole/endosome tethering (CORVET) complexes (Mackie et al., 2018). Furthermore, preliminary data from our lab has identified α-arrestin-mediated trafficking of Kir1.1.
Table 1. Summary of Kir2.x disease causing mutations linked to protein trafficking.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Gene</th>
<th>Aliases</th>
<th>Localization</th>
<th>Disease</th>
<th>Trafficking Mutations</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kir2.1</td>
<td>KCNJ2</td>
<td>IRK1</td>
<td>Heart, smooth muscle, skeletal muscle, placenta, kidney</td>
<td>Andersen-Tawil Syndrome (Long QT syndrome)</td>
<td>S314-Y315; S369X; A95-98; V302M*; C101R; T75M; G52V; G300V*; E300K*; T74A*; R218W</td>
<td>(Ambrosini et al., 2014; Ballester et al., 2006; Bendahhou et al., 2003; de Boer et al., 2010; Doi et al., 2011; Gelinas et al., 2017; Lopes et al., 2002; Ma et al., 2011; Tani et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LQT7</td>
<td></td>
<td></td>
<td>Short QT syndrome</td>
<td>K346T</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IRK-1</td>
<td></td>
<td></td>
<td>Familial atrium fibrillation</td>
<td>-</td>
</tr>
<tr>
<td>Kir2.2</td>
<td>KCNJ12</td>
<td>IRK2</td>
<td>brain, eye, heart, smooth muscle, skeletal muscle, kidney</td>
<td>Familial Esophageal Squamous Cell Carcinoma</td>
<td>-</td>
<td>(de Boer et al., 2010; Khalilipour et al., 2018)</td>
</tr>
<tr>
<td>Kir2.3</td>
<td>KCNJ4</td>
<td>HIRK2</td>
<td>brain, eye, heart, smooth muscle</td>
<td>Parkinson’s disease</td>
<td>-</td>
<td>(de Boer et al., 2010; Shen et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IRK3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HIR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kir2.4</td>
<td>KCNJ14</td>
<td>IRK4</td>
<td>brain, eye, heart, smooth muscle</td>
<td>-</td>
<td>-</td>
<td>(de Boer et al., 2010)</td>
</tr>
<tr>
<td>Kir2.6</td>
<td>KCNJ18</td>
<td>TTPP2</td>
<td>Skeletal muscle</td>
<td>Thyrotoxic hypokalemic periodic paralysis</td>
<td>R399X*; Q407X*; R43C; A200P</td>
<td>(Cheng et al., 2011; de Boer et al., 2010; Ryan et al., 2010)</td>
</tr>
</tbody>
</table>

*Denotes mutations that are suspected to impair protein trafficking, but are yet to be fully defined.
1.2 Protein trafficking and the α-arrestins

Protein composition at the plasma membrane (PM) must be tightly regulated to ensure cell health. This involves continuous protein internalization along with selective targeting to the cell surface in response to environmental changes. This selective trafficking can be seen with ion channels, like Kir2.1 in response to physiological changes. There are many components involved in selective protein trafficking, but our interest in protein trafficking primarily focuses on a family of protein trafficking adaptors known as the α-arrestins.

1.2.1 Role of α-arrestins in endocytosis in yeast and mammals

One-way cells effectively respond to their environment is through regulated endocytosis. Endocytosis is the mechanism in which membrane vesicles are produced de novo from the plasma membrane (Doherty and McMahon, 2009). This method allows the cell to selectively remove plasma membrane proteins in response to environmental changes. Our work will help discover mechanistic details in the endocytic pathway. We have a detailed understanding of the molecular players in this pathway, with spatial and temporal resolution for >60 proteins in clathrin-mediated endocytosis (CME) (Kirchhausen, 2000a). CME involves clathrin assembly at the PM via clathrin adaptor proteins (AP) that help bridge clathrin polyhedral lattices to transmembrane proteins (Kirchhausen, 2000a, b; Sorkin, 2004).

Studies of α-arrestin function are just beginning, with most of the work on these regulators done in budding yeast. First identified in 2008, the α-arrestins arise earlier in cells (i.e., present in prokaryotes and eukaryotes) and are more broadly conserved across organisms when compared to their well-studied and closely related β-arrestins, which are only present in multi-cellular
eukaryotes (Alvarez, 2008). The β-arrestins are most notably known for their role in the internalization of G-protein coupled receptors (GPCR) via the AP-2 adaptin complex and CME (Bircsak et al., 2018; Miller and Lefkowitz, 2001; Shenoy and Lefkowitz, 2011). Crystal structures and secondary predictions reveal that the arrestin family members have conserved arrestin-fold domains in their N- and C-terminal that consist of 7 anti-parallel beta sheets (Granzin et al., 2012; Han et al., 2001; Hwang et al., 2014; Shi et al., 2006). However, although both subfamilies are highly conserved, nearly all α-arrestins possess a L/PPxY motif in their C-terminal tail that is absent in β-arrestins (Alvarez, 2008). In yeast, these L/PPxY motifs allow for the α-arrestins to bind the WW-domains on the ubiquitin ligase Rsp5, the mammalian ortholog of which is Nedd4 (neural precursor development downregulated protein 4) homolog (Figure 3A) (Lin et al., 2008; Rotin et al., 2000). The ability of the α-arrestins to bind Rsp5 is required for α-arrestin-mediated trafficking of membrane proteins (Lin et al., 2008; Nikko and Pelham, 2009; O'Donnell et al., 2010). This knowledge stems from early experiments that employed a genetic screen to identify genes involved in endocytosis. Specifically, Lin et al. took advantage of the S. cerevisiae knockout collection, a the collection of 4,652 strains that have each of the nonessential genes deleted, and tested for sensitivity to a toxic arginine analog, canavanine, that uses the arginine transporter, Can1, to enter the cell (Lin et al., 2008). They hypothesized that increased sensitivity to canavanine would be due to inhibition of endocytosis and thus increased PM localization of Can1. This screen identified an uncharacterized open reading frame YOR322C, which they refer to as cvs7 and is known today as the α-arrestin Art1/Ldb19, which regulates Can1 endocytosis and when deleted causes retention of Can1 at the PM (Lin et al., 2008). They identified the L/PPxY (also referred to as PPXY) motifs via bioinformatics analysis, which they hypothesized to bind with the WW domains of Rsp5. They assessed the interaction between Art1/Ldb19 and the WW domains of Rsp5 by measuring in vitro
binding of GST fused to the WW domains of Rsp5 to \textit{in vitro}-translated Art1/Ldb19 (Lin et al., 2008). Their results confirm \(\alpha\)-arrestin Art1/Ldb19 as a ubiquitin ligase adaptor that acts a nexus between Rsp5 and Can1, allowing Rsp5 to ubiquitinate Can1 and induce endocytosis. This interaction is similar between the mammalian \(\alpha\)-arrestins and Nedd4-family ubiquitin ligases, where this interaction is required for normal \(\alpha\)-arrestin-mediated trafficking (Han et al., 2013; Nabhan et al., 2010). This was revealed while studying the prolonged stimulation of the \(\beta\)2-adrenergic receptor (\(\beta\)2AR) in HEK293T cells (Nabhan et al., 2010). Specifically, Nabhan \textit{et al.} found that \(\beta\)2AR was ubiquitinated after prolonged stimulation by isoproterenol (ISO) and using a genome-wide RNA interference screen they were able to identify the mammalian \(\alpha\)-arrestin, ARRDC3, as essential for this downregulation (Nabhan et al., 2010). Similar to studies in yeast, they found that the PPXY motifs in ARRDC3 interacts with the WW domains within the ubiquitin ligase Nedd4 (Nabhan et al., 2010). This direct interaction was shown using both co-immunoprecipitations and co-localization studies wherein they generated double PPXY mutants that were unable to interact or co-localize with Nedd4.

There are currently fourteen yeast \(\alpha\)-arrestins (Ldb19/Art1, Ecm21/Art2, Aly2/Art3, Rod1/Art4, Art5, Aly1/Art6, Rog3/Art7, Csr2/Art8, Rim8/Art9, Art10, Bul1, Bul2, and Spo23) and 6 mammalian \(\alpha\)-arrestins (\texttt{Arrestin-Domain Containing Proteins} (ARRDC1, ARRDC2, ARRDC3, ARRDC4, ARRDC5), and \texttt{Thioredoxin Interacting Protein 1} (TXNIP)). Since their initial discovery in 2008, many plasma membrane proteins (referred to hereafter as “cargos”) have been shown to be controlled by the \(\alpha\)-arrestins in both yeast and mammalian cells (reviewed in O’Donnell and Schmidt (O’Donnell and Schmidt, 2019). In short, these cargos include ion channels, glucose transporters, amino acid permeases, and G-protein coupled receptors (GPCRs)
(O'Donnell and Schmidt, 2019) and it is likely that additional cargoes will be identified in the coming years.

As indicated earlier the α-arrestins were first identified to aid in endocytosis of cargos and much of the earlier work detailing their function focused on this role alone. Notable examples where α-arrestins aid in the endocytosis of cargo include Bul1 and Bul2 promoting the endocytosis of Gap1 (Merhi and Andre, 2012); Ldb19-mediated endocytosis of Can1 and Mup1; Art2 promotion of endocytosis of Lyp1 (Lin et al., 2008); and Aly1 and Aly2-mediated endocytosis of Dip5 (O'Donnell et al., 2013). While the α-arrestins role in endocytosis is well studied and verified, there is more recent evidence that these trafficking adaptors are involved in the intracellular sorting and recycling of select cargo and I detail these examples in the following section.

1.2.2 Role of α-arrestins in intracellular sorting and recycling

Inside the cell, vesicles are continuously trafficking proteins between membrane-bound compartments. The pathways used are often associated with intracellular sorting, which involves the direct trafficking of proteins from the Golgi to endosomes to vacuoles, or the recycling pathway, which involves the trafficking of proteins from the PM through either the endosomes or Golgi and back to the PM. Early studies of intracellular trafficking were done using pulse-chase experiments and electron microscopy (Palade, 1975). Later, studies using yeast genetics exploited specific gene mutations to uncover trafficking pathways and identify the genes that were involved (Schekman, 1992). These early studies and many others have elucidated the mechanistic details behind intracellular trafficking. In short, proteins are trafficked from the ER to the Golgi while going through post translational modification such as the addition of sugars and/or lipids. As a
protein matures through the TGN, they undergo quality control and are either returned to the ER or continue to membrane compartments like the plasma membrane or endosomes (Sun and Brodsky, 2019). Proteins that are shuffled to the endosomes have three potential further pathways: 1) traffic to the vacuole for degradation, 2) traffic back to the TGN, or 3) transport out to the PM via exosomes.

The role that α-arrestins play in endocytosis of nutrient and other transporters in yeast is well-described as mentioned previously (Becuwe et al., 2012; Hatakeyama et al., 2010; Lin et al., 2008; Merhi and Andre, 2012; Nikko and Pelham, 2009; O'Donnell et al., 2015; Rotin and Kumar, 2009). However, there are several studies that demonstrate α-arrestins also regulate intracellular sorting (Table 2). For example, studies on the α-arrestins, Bul1 and Bul2, which predate these proteins being described as α-arrestins, demonstrate a clear role in the intracellular trafficking of Gap1 (Crapeau et al., 2014; Helliwell et al., 2001; Merhi and Andre, 2012; Soetens et al., 2001). Gap1 is the general amino acid permease in yeast and can uptake a broad range of amino acids (Grenson et al., 1970). Thus Gap1 is a critical protein when cells experience nitrogen starvation (Courchesne and Magasanik, 1983; Grenson et al., 1970; Stanbrough and Magasanik, 1995). For example, when cells undergo nitrogen starvation, Gap1 is localized to the PM to replace specific amino acid permeases that are typically expressed under normal growth conditions. The cell replaces these specific amino acid permeases with Gap1 in order to cut back on the energy being expended to manufacture and regulate multiple permeases (De Craene et al., 2001; Grenson et al., 1970; Jauniaux and Grenson, 1990). As one would expect, when the cell is then given its preferred source of nitrogen, such as ammonium or glutamate, Gap1 is no longer needed and is downregulated via its ubiquitination and endocytosis (Hein et al., 1995; Soetens et al., 2001; Springael and Andre, 1998). Under these conditions, Gap1 is not only endocytosed but also
undergoes sorting to the vacuole from the Golgi and endosomes (Soetens et al., 2001). Interestingly, the intracellular sorting of Gap1 does not require it to reach the PM, however, it does need to be ubiquitinated by Bul1/Bul2 α-arrestins (Helliwell et al., 2001; Soetens et al., 2001). Further studies revealed that this nitrogen dependence activity of Bul1 and Bul2-mediated-ubiquitination of Gap1 relies on phosphorylation and dephosphorylation of the Buls by Npr1 and Sit4 respectively. Npr1 is a (nitrogen permease reactivator 1) kinase that is essential for Gap1 activity (Grenson, 1983) and Sit4 is a protein phosphatase that is regulated by TORC1 and dephosphorylates Npr1 (Jacinto et al., 2001). When Bul1/Bul2 are phosphorylated, they are inhibited via an interaction with 14-3-3 proteins (Merhi and Andre, 2012). 14-3-3 proteins bind to phosphorylated sites, altering protein conformation and resulting in a wide range of regulatory functions (Bridges and Moorhead, 2005). When cells are growing on a poor-quality nitrogen source, like proline, Gap1 is localized to the cell surface. Additionally, Bul1 and Bul2 are phosphorylated by Npr1 and then bound to 14-3-3 proteins. This inhibits their interaction with Rsp5 and thereby blocks Gap1 ubiquitination. However, when ammonium is present, Gap1 is not needed at the cell surface. In these conditions, Npr1 is inactivated and Bul1 and Bul2 can be dephosphorylated by Sit4, thus dissociating from 14-3-3 proteins, and are now able to bind to and ubiquitinate Gap1. This then stimulates the endocytosis and intracellular sorting of Gap1 to the vacuole for degradation (Merhi and Andre, 2012).

When cells are treated with TORC1 inhibitor rapamycin, Gap1 is constitutively downregulated. Both Bul1 and Bul2 promote Gap1 downregulation without undergoing dephosphorylation and do so via the C-terminal region that is not associated with ubiquitination in response to internal amino acid levels (Crapeau et al., 2014). More recent studies show that in response to nutrients, additional α-arrestins, Aly1 and Aly2, also control the intracellular sorting
of Gap1. Specifically, O’Donnell et al. found that Aly2-dependent trafficking of Gap1 required AP-1 and phosphorylation by Npr1 (O’Donnell et al., 2010). AP-1 is a clathrin adaptor complex that is involved in endosome-to-Golgi transport. This is consistent with the findings of Bul1 and Bul2 as stated above (Crapeau et al., 2014; O’Donnell et al., 2010). Since phosphorylation of the α-arrestins played a role in previous studies (Becuwe et al., 2012; MacGurn et al., 2011; Merhi and Andre, 2012; O’Donnell et al., 2010), its role here in Gap1 intracellular trafficking was examined. Specifically, phosphorylation is like a switch that controls the α-arrestins’ role in either intracellular sorting or endocytosis (Figure 3, B and C). When the calcineurin binding site on Aly1 was mutated to prevent calcineurin binding there was no effect on Aly1-mediated Gap1 intracellular trafficking, (O’Donnell et al., 2013) suggesting that dephosphorylation of the α-arrestins was not needed for intracellular trafficking of Gap1.

The role for α-arrestins in intracellular sorting is further supported by studies of α-arrestin Rod1’s regulation of Jen1, a monocarboxylate transporter (Becuwe and Leon, 2014). Rod1 function appears to be two-fold; it acts as a regulator of Jen1 endocytosis and recycling depending on its location within the cell. When recruited to the plasma membrane, Rod1 drives endocytosis, but when localized to the trans-Golgi networks Rod1 stimulates intracellular sorting between the Golgi and endosomes (Becuwe and Leon, 2014; Becuwe et al., 2012; Hovsepian et al., 2018). Specifically, when Rod1 was deleted Jen1 was internalized but unable to reach the endosome and instead was relocalized to the PM suggesting that Rod1 is not essential for Jen1 endocytosis, but still plays a strong role in Jen1 intracellular sorting. Interestingly, it appears that Bul1 is responsible for Jen1 internalization (Hovsepian et al., 2018). Additionally, Rod1 was translocated to the TGN in response to glucose in a Reg1-dependent manner (Becuwe and Leon, 2014). Reg1 is a regulatory subunit of the protein phosphatase 1 (PP1) that interacts with Rod1 and is needed for the
ubiquitination and activation of Rod1 via dephosphorylation (Becuwe et al., 2012). Consequently, in a Rod1 deletion, Jen1 accumulates at the TGN and does not make it to the vacuole. Further experiments were done by deleting retrograde or endosome-to-TGN trafficking components to uncover what pathways are used by Jen1. Deletions of the yeast Rab6 homologue \((ypt6\Delta)\) and its guanine nucleotide exchange factor or GEF \((rpg1\Delta \text{ and } ric1\Delta)\) (Siniosoglou et al., 2000) stopped Jen1 from localizing to the TGN suggesting that Jen1 localizes to the TGN after endocytosis before being delivered to the vacuole (Becuwe and Leon, 2014). When the Golgi-localized clathrin adaptors, Gga1 and Gga2, that traffic from the TGN to the endosomes were deleted, Jen1 targeting to the vacuole was impaired and this gave rise to Jen1 recycling to the PM (Becuwe and Leon, 2014). When Jen1 was internalized but not degraded, it became deubiquitinated. When endocytosis is blocked using a deletion of Vrp1, which inhibits endocytosis by preventing actin nucleation, Jen1 was robustly ubiquitinated, suggesting that along the route from the PM to the Golgi, Jen1 becomes deubiquitinated (Becuwe and Leon, 2014). When Jen1 is retained at the Golgi it becomes poly-ubiquitinated. Additionally, at the Golgi, Jen1 co-localizes with Rod1 and Rsp5. This suggests that Rod1 at the Golgi may allow for Jen1 to be re-ubiquitinated by Rsp5 and thus promotes trafficking from the TGN to the vacuole (Becuwe and Leon, 2014).

Supporting the data above, \(\alpha\)-arrestins localize to a number of subcellular locations (Becuwe and Leon, 2014; Lin et al., 2008; MacGurn et al., 2011; Martinez-Marquez and Duncan, 2018; O'Donnell et al., 2010). Specifically, \(\alpha\)-arrestins Ldb19 localizes to the Golgi under basal conditions and re-localizes to the PM under stress (MacGurn et al., 2011; Martinez-Marquez and Duncan, 2018). Rod1 localizes to the Golgi, as mentioned above (Becuwe and Leon, 2014). Martinez-Marquez et al. showed that Ldb19 co-localizes with late stages of TGN maturation and using bimolecular fluorescence complementation (BiFC) identified an interaction between Ldb19
and the adaptor complex 1 (AP-1) (Martinez-Marquez and Duncan, 2018). Furthermore, when Ldb19 was hypophosphorylated it accumulated extensively at the plasma membrane, but when Ldb19 was hyperphosphorylated it localized primarily to the cytosol and Golgi (MacGurn et al., 2011). This suggests that Ldb19 localization is phosphor-regulated and that it can control alternative trafficking pathways dependent upon its localization (MacGurn et al., 2011). Together these data give strong evidence supporting a role for the α-arrestins in intracellular sorting and set the stage for work detailed in Chapter 2 where we show select α-arrestins promote cell surface localization of the mammalian potassium channel Kir2.1.

Figure 3. α-Arrestin summary
(A) Schematic relationship between α-arrestins and the ubiquitin ligase Rsp5. (B) Phosphorylation as a switch between α-arrestin role in intracellular sorting and endocytosis. (C) Schematic displaying the intracellular pathway and endocytic pathway.

Table 2. Yeast α-arrestins and their cargo

<table>
<thead>
<tr>
<th>α-Arrestin</th>
<th>Endocytosis Cargo</th>
<th>Intracellular Sorting Cargo</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Art1/Ldb19</td>
<td>Mup1, Ste2, Ste3, Can1, Lyp1, Tat2, Fur4</td>
<td>Kir2.1</td>
<td>(Alvaro et al., 2014; Hager et al., 2018; Nikko and Pelham, 2009; Prosser et al., 2015)</td>
</tr>
<tr>
<td>Art2/ECM21</td>
<td>Tat2, Fur4, Lyp1, Smf1</td>
<td>-</td>
<td>(Nikko and Pelham, 2009)</td>
</tr>
<tr>
<td>Art3/Aly2</td>
<td>Dip5, Ste3, Git1</td>
<td>Gap1, Kir2.1</td>
<td>(Crapeau et al., 2014; Hager et al., 2018; Hatakeyama et al., 2010; O'Donnell et al., 2010; O'Donnell et al., 2013; Prosser et al., 2015; Robinson et al., 2022)</td>
</tr>
<tr>
<td>Art4/Rod1</td>
<td>Hxt1, Hxt3, Jen1</td>
<td>Jen1</td>
<td>(Becuwe et al., 2012; Nikko and Pelham, 2009; O'Donnell et al., 2015)</td>
</tr>
<tr>
<td>Art5</td>
<td>Itr1</td>
<td>-</td>
<td>(Nikko and Pelham, 2009)</td>
</tr>
<tr>
<td>Art6/Aly1</td>
<td>Dip5, Ste3</td>
<td>Gap1, Kir2.1</td>
<td>(Crapeau et al., 2014; Hager et al., 2018; O'Donnell et al., 2010; O'Donnell et al., 2013; Prosser et al., 2015)</td>
</tr>
<tr>
<td>α-Arrestin</td>
<td>Endocytosis Cargo</td>
<td>Intracellular Sorting Cargo</td>
<td>References</td>
</tr>
<tr>
<td>------------</td>
<td>-------------------</td>
<td>-----------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>Art7/Rog3</td>
<td>Hxt3</td>
<td>-</td>
<td>(O'Donnell et al., 2015)</td>
</tr>
<tr>
<td>Art8/Csr2/Mrg19</td>
<td>Hxt6, Hxt7, Hxt2, Hxt4</td>
<td>-</td>
<td>(Hovsepian et al., 2017; Snowdon and van der Merwe, 2012)</td>
</tr>
<tr>
<td>Art9/Rim8</td>
<td>Rim21, Pma1</td>
<td>-</td>
<td>(Gomez-Raja and Davis, 2012; Herrador et al., 2015; Smardon and Kane, 2014)</td>
</tr>
<tr>
<td>Art10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bul1/Smm2/Dag1/Rds1</td>
<td>Jen1, Ptr2, Tat1, Tat2, Ctr1, Put4, Dal5</td>
<td>Gap1</td>
<td>(Abe and Iida, 2003; Hovsepian et al., 2018; Kawai et al., 2014; Liu et al., 2007; Merhi and Andre, 2012; Suzuki et al., 2013; Villers et al., 2017)</td>
</tr>
<tr>
<td>Bul2</td>
<td>Ptr2, Tat1, Tat2, Ctr1, Put4, Dal5</td>
<td>Gap1</td>
<td>(Abe and Iida, 2003; Liu et al., 2007; Merhi and Andre, 2012; Suzuki et al., 2013; Villers et al., 2017)</td>
</tr>
<tr>
<td>Bul3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Spo23</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
2.0 Select α-Arrestins Control the Cell-Surface Abundance of the Mammalian Kir2.1 Potassium Channel in a Yeast Model

The contents of this chapter are adapted from a published article in the *Journal of Biological Chemistry* (*Hager et al.*, 2018). N.A.H. completed Figure 5A, 7B, 7C, 7D, 7E, 7F, 9C, 9D, 11B, 11E, 12B, 12C, 12D, and 12E.

2.1 Introduction

In order to accurately respond to a cell’s environment, many proteins are delivered to or removed from the cell surface or redistributed between intracellular membranes. This method of reshuffling proteins between membranes is commonly referred to as protein trafficking and is highly selective for specific membrane proteins. As discussed in Chapter 1, selective protein trafficking is essential for optimal cell health, especially for human cardiac and muscle health; Every heartbeat is dependent on accurate selective protein trafficking of different ion channels to ensure ion balance and heart rhythm is maintained. Although human health is reliant on the correct protein trafficking of these ion channels, there is still much to be discovered. In part, this advancement of knowledge is hindered due to the complexity and challenges of studying heart muscle cells (*Brette and Orchard*, 2003; *Curran et al.*, 2014). To overcome this obstacle we and others have taken advantage of a yeast model system to study ion channels (*Ko and Gaber*, 1991; *Kolb et al.*, 2014). Specifically, here we use yeast to identify factors needed for the protein trafficking of the inward rectifying potassium channel, Kir2.1. In our yeast system, the two
endogenous yeast potassium channels, Trk1 and Trk2, are deleted and in their place the mammalian potassium channel Kir2.1, originally defined as IRK1, is ectopically expressed (Kolb et al., 2014; Tang et al., 1995). This system has successfully been used to reveal information regarding various potassium channel function and regulation (Mackie and Brodsky, 2018).

Kir2.1 is a member of the family of inward rectifying K\(^+\) channels and expressed in the heart, skeletal muscle, and neurons where it is responsible for maintaining membrane potential (de Boer et al., 2010; Nichols and Lopatin, 1997). As stated previously, Kir2.1 is of medical interest due to its role in various diseases including short or long QT syndrome, which results in cardiac arrhythmias (Plaster et al., 2001; Priori et al., 2005). Understanding the trafficking of Kir2.1 is essential to correcting variations of this diseased-state as several of the disease-causing mutations are tied to Kir2.1 trafficking as covered previously in Chapter 1. Most notably, deletion of amino acids 314-315 inhibits the ability of Kir2.1 to bind to the clathrin adaptin complex 1 (AP-1), which traffics Kir2.1 from the Golgi to the PM (Ma et al., 2011). With this in mind, our collaborators conducted a screen where they identified regulators of Kir2.1, and unsurprisingly, 68% of their hits were involved in protein trafficking (Kolb et al., 2014). More specifically, this screen crossed the trk1\(\Delta\) trk2\(\Delta\) strain with the yeast deletion collection that has each of the nonessential genes deleted and ecopically expressed Kir2.1 into each of these newly created strains. Then they did serial dilution assays (spot assays) on plates with varying concentrations of potassium and assessed growth. From this assay they were able to generate a list of Kir2.1 regulators of which 37 out of 54 candidates were involved in protein trafficking (Kolb et al., 2014). However, due to this method of testing for regulators, any redundant regulators were likely absent from their list of hits.

In this Chapter we used a more targeted screen focusing on the role \( \alpha\)-arrestins play in Kir2.1 trafficking in yeast. As stated previously in Chapter 1, \( \alpha\)-arrestins are a family of protein
trafficking adaptors that are involved in directing membrane proteins or cargo. In short, α-arrestins link specific cargo proteins to the ubiquitin ligase Rsp5, which facilitates protein sorting in the secretory pathway (Alvaro et al., 2014; Becuwe et al., 2012; Lin et al., 2008; Nikko and Pelham, 2009; Nikko et al., 2008; O'Donnell et al., 2013). In our target screen, we were able to identify three α-arrestins—Aly1, Aly2, and Ldb19 (also known as Art6, Art3, and Art1, respectively)—that regulate Kir2.1 trafficking. Specifically, these α-arrestins promote Kir2.1-dependent growth on low potassium medium, increase Kir2.1 localization at the PM, and increase intracellular potassium levels. Additionally, we show that Rsp5 is required for this α-arrestin-mediated trafficking of Kir2.1 and that the ability of Aly1 to regulate Kir2.1 trafficking is regulated by phosphorylation.

Together, these findings set the stage for future studies to be done analyzing the role of α-arrestin-mediated trafficking of Kir2.1 in human cells such as cardiomyocytes, and identify a new cargo under the control of the α-arrestins.

2.2 Materials and Methods

2.2.1 Yeast strains and growth conditions

Yeast strains used in this study and their construction are described in Table 3. Yeast were grown in synthetic complete (SC) medium prepared as described (Johnston et al., 1977). SC low-potassium medium was also prepared as described with the use of monosodium glutamate as a nitrogen source, the addition of 20 mM MES to maintain the pH, and the indicated amount of KCl (Kolb et al., 2014). Liquid medium was filter-sterilized, and for plated medium, 2% (w/v) agar
was added prior to autoclaving. Plasmids were transformed into yeast via the lithium-acetate method and selected for on appropriate SC medium. Yeast cells were grown at 30°C unless otherwise indicated. For growth assays on solid medium, 5-fold serial dilutions of saturated, overnight cultures (starting concentration of $1.0 \times 10^7$ cells/ml) were plated onto the indicated medium and grown for 3-6 days at 30°C.

**Table 3. Yeast strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4741</td>
<td>$MATa\ his3\Delta 1\ leu2\Delta 0\ ura3\Delta 0\ met15\Delta 0$</td>
<td>(Brachmann et al., 1998)</td>
</tr>
<tr>
<td>BY4743</td>
<td>$MATa/MATa\ his3\Delta 1/his3\Delta 1\ leu2\Delta 0/leu2\Delta 0\ ura3\Delta 0/ura3\Delta 0\ met15\Delta 0/MET15\ LYS2/lys\Delta 0$</td>
<td>(Brachmann et al., 1998)</td>
</tr>
<tr>
<td>trk1∆ trk2∆</td>
<td>$MAT\alpha\ trk1\Delta::HIS3\ trk2\Delta::HIS3\ his3\Delta 200\ leu2\Delta 1\ ura3-52\ trp1-1\ ade2$</td>
<td>(Ko and Gaber, 1991; Nakamura and Gaber, 1998)</td>
</tr>
<tr>
<td>Query strain trk1∆ trk2∆ (YAK01)</td>
<td>$MAT\alpha\ trk1\Delta::URA3\ trk2\Delta::NAT\ his3\Delta 1\ leu2\Delta 0\ ura3\Delta 0\ lyp1\Delta 0\ lys\Delta 0\ can1\Delta::STE2pr-HIS3$</td>
<td>(Kolb et al., 2014)</td>
</tr>
<tr>
<td>trk1∆ trk2∆ vps35∆</td>
<td>$MATa\ trk1\Delta::URA3\ trk2\Delta::NAT\ vps35\Delta::KAN\ his3\Delta 1\ leu2\Delta 0\ ura3\Delta 0\ lyp1\Delta 0\ lys\Delta 0\ can1\Delta::STE2pr-HIS3$</td>
<td>This study. This strain was derived from YAK01. VPS35 coding region was replaced by the KAN cassette as described in Ref (Longtine et al., 1998).</td>
</tr>
<tr>
<td>trk1∆ trk2∆ cnb1∆</td>
<td>$MAT\alpha\ trk1\Delta::HIS3\ trk2\Delta::HIS3\ cnb1\Delta::HPH\ his3\Delta 200\ leu2\Delta 1\ ura3-52\ trp1-1\ ade2$</td>
<td>This study. This strain was derived from the trk1∆ trk2∆ (Ko and Gaber, 1991; Nakamura and Gaber, 1998). The CNB1 coding region was replaced by the hygromycin resistance cassette as described in (Ko and Gaber, 1991).</td>
</tr>
<tr>
<td>HRD1/DOA10 (WT)</td>
<td>$MAT\alpha\ ade2\ his3\ leu2\ ura3\ trp1$</td>
<td>(Kolb et al., 2014; Pagant et al., 2007)</td>
</tr>
<tr>
<td>pdr5∆</td>
<td>$MAT\alpha\ pdr5\Delta::KAN\ his3\Delta 1\ leu2\Delta 0$</td>
<td>(Winzeler et al., 1999).</td>
</tr>
<tr>
<td>BJ5459</td>
<td>$MATa\ his3\Delta 200\ leu2\Delta 1\ ura3-52\ trp1\ lys2-801\ pep4\Delta::HIS3\ prb1\Delta 1.6R\ can1\ GAL$</td>
<td>(Jones, 1991)</td>
</tr>
</tbody>
</table>
2.2.2 Plasmids and DNA manipulations

Plasmids used in this study and details of their construction are described in Table 4. PCR amplifications were performed using Phusion High Fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, MA), and all constructs generated were verified using Sanger sequencing (Genewiz, South Plainfield, NJ).

Table 4. Plasmids used in Chapter 2

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Description/References</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRS415-TEF1pr</td>
<td>TEF1pr CEN LEU2</td>
<td>(Mumberg et al., 1995)</td>
</tr>
<tr>
<td>pRS415-TEF1pr-Kir2.1-HA</td>
<td>TEF1pr-Kir2.1-HA CEN LEU2</td>
<td>(Kolb et al., 2014)</td>
</tr>
<tr>
<td>pRS415-TEF1pr-FAP-TM-Kir2.1-HA</td>
<td>TEF1pr-MFal1-252-FAP-TM-Kir2.1-HA CEN LEU2</td>
<td>The pRS415-TEF1pr-Kir2.1-HA plasmid was used as a starting point to insert the MFal (nucleotides 1-252) expressing the first 84 amino acids needed for targeting the protein to the ER. This region of MFal was PCR amplified from genomic DNA using primers with SpeI and BamHI restriction enzyme adaptors. The FAP-TM was PCR amplified from pBABE-kappa-myc-dL5-G4S-TMst plasmid (Kolb et al., 2014) so that just the dL5-G4S-TM domain was contained between BamHI and SmaI restriction enzyme site adaptors. It should be noted that the TM comes from the platelet-derived growth factor receptor. The MFal and FAP-TM were cloned in frame at the N-terminus of Kir2.1. The plasmid was confirmed by sequence analyses.</td>
</tr>
<tr>
<td>pRS415-TEF1pr-Kir2.1-AAA-HA</td>
<td>TEF1pr-Kir2.1GTYG-AAA-HA CEN LEU2</td>
<td></td>
</tr>
<tr>
<td>pRS426-Kir2.1</td>
<td>GPD1pr-Kir2.1-HA 2 μm URA3</td>
<td>(Kolb et al., 2014)</td>
</tr>
<tr>
<td>pRS425</td>
<td>2 μm LEU2</td>
<td>(Sikorski and Hieter, 1989)</td>
</tr>
<tr>
<td>pRS425-ALY1</td>
<td>ALY1pr-ALY1 2 μm LEU2</td>
<td>(Prosser et al., 2015)</td>
</tr>
<tr>
<td>pRS426</td>
<td>2 μm URA3</td>
<td>(Sikorski and Hieter, 1989)</td>
</tr>
<tr>
<td>pRS426-ALY1</td>
<td>ALY1pr-ALY1 2 μm URA3</td>
<td>(O’Donnell et al., 2010)</td>
</tr>
<tr>
<td>pRS426-ALY2</td>
<td>ALY2pr-ALY2 2 μm URA3</td>
<td>(O’Donnell et al., 2010)</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Genotype</td>
<td>Description/References</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>pRS426-LDB19</td>
<td>LDB19pr-LDB19 2 μ</td>
<td>(Alvaro et al., 2014)</td>
</tr>
<tr>
<td>pRS426-ROD1</td>
<td>ROD1pr-ROD1 2 μ URA3</td>
<td>(Alvaro et al., 2014)</td>
</tr>
<tr>
<td>pRS426-ROG3</td>
<td>ROG3pr-ROG3 2 μ URA3</td>
<td>(O'Donnell et al., 2010)</td>
</tr>
<tr>
<td>pRS426-CSR2</td>
<td>CSR2pr-CSR2 2 μ URA3</td>
<td>(Prosser et al., 2015)</td>
</tr>
<tr>
<td>pRS426-ECM21</td>
<td>ECM21pr-ECM21 2 μ URA3</td>
<td>(Prosser et al., 2015)</td>
</tr>
<tr>
<td>pRS426-ART5</td>
<td>ART5pr-ART5 2 μ URA3</td>
<td>(Prosser et al., 2015)</td>
</tr>
<tr>
<td>pRS426-ART10</td>
<td>ART10pr-ART10 2 μ URA3</td>
<td>(Prosser et al., 2015)</td>
</tr>
<tr>
<td>pRS426-RIM8</td>
<td>RIM8pr-RIM8 2 μ URA3</td>
<td>(Prosser et al., 2015)</td>
</tr>
<tr>
<td>pRS426-BUL1</td>
<td>BUL1pr-BUL1 2 μ URA3</td>
<td>(Prosser et al., 2015)</td>
</tr>
<tr>
<td>pRS426-BUL2</td>
<td>BUL2pr-BUL2 2 μ URA3</td>
<td>(Prosser et al., 2015)</td>
</tr>
<tr>
<td>pRS426-aly1PPXYLESS</td>
<td>ALY1pr-aly1PPXYLESS 2 μ URA3</td>
<td>(Prosser et al., 2015)</td>
</tr>
<tr>
<td>pRS426-aly2PPXYLESS</td>
<td>ALY2pr-aly2PPXYLESS 2 μ URA3</td>
<td>(Prosser et al., 2015)</td>
</tr>
<tr>
<td>pRS426-lldb19PPXYLESS</td>
<td>LDB19pr-lldb19PPXYLESS 2 μ URA3</td>
<td>(Prosser et al., 2015)</td>
</tr>
<tr>
<td>pRS426-aly1AAAAA</td>
<td>ALY1pr-aly1AAAAA 2 μ URA3</td>
<td>(O'Donnell et al., 2013)</td>
</tr>
<tr>
<td>pRS426-aly1K379R</td>
<td>ALY1pr-aly1K379R 2 μ URA3</td>
<td>This plasmid was made using site-directed mutagenesis primers that converted the lysine at 379 to an arginine. The pRS426-ALY1 plasmid was used as a template.</td>
</tr>
<tr>
<td>pRS426-aly2K392R</td>
<td>ALY2pr-aly2K392R 2 μ URA3</td>
<td>This plasmid was made using site-directed mutagenesis primers that converted the lysine at 392 to an arginine. The pRS426-ALY1 plasmid was used as a template.</td>
</tr>
<tr>
<td>pKK212-ALY1</td>
<td>CUP1pr-ALY1 2 μ TRP1</td>
<td>(O'Donnell et al., 2010)</td>
</tr>
<tr>
<td>pKK212-ALY2</td>
<td>CUP1pr-ALY1 2 μ TRP1</td>
<td>(O'Donnell et al., 2010)</td>
</tr>
<tr>
<td>pKK212-aly1PPXYLESS</td>
<td>CUP1pr-aly1PPXYLESS 2 μ TRP1</td>
<td>pRS426-ALY1PPXYLESS (Prosser et al., 2015) was PCR amplified using primers that amplified the coding region of Aly1PPXYLESS and contained XmaI and SalI restriction enzyme adaptors. This was cloned into pKK212 to create an N-terminal, in-frame GST fusion.</td>
</tr>
<tr>
<td>pKK212-aly1K392R</td>
<td>CUP1pr-aly1K392R 2 μ TRP1</td>
<td>pRS426-ALY2pr-aly1K392R (this study) was PCR amplified using primers that amplified the coding region of Aly2K392R and contained XmaI and SalI restriction enzyme adaptors. This was cloned into pKK212 to create an N-terminal, in-frame GST fusion.</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Genotype</td>
<td>Description/References</td>
</tr>
<tr>
<td>--------------------------</td>
<td>----------------------------</td>
<td>----------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>pKK212-aly2&lt;sup&gt;PPXYLESS&lt;/sup&gt;</td>
<td>CUP1pr- aly2&lt;sup&gt;PPXYLESS&lt;/sup&gt; TRP1</td>
<td>2μ pRS426-aly2&lt;sup&gt;PPXYLESS&lt;/sup&gt; (Prosser et al., 2015) was PCR amplified using primers that amplified the coding region of Aly2&lt;sup&gt;PPXYLESS&lt;/sup&gt; and contained XmaI and SalI restriction enzyme adaptors. This was cloned into pKK212 to create an N-terminal, in-frame GST fusion.</td>
</tr>
<tr>
<td>pKK212-aly2&lt;sup&gt;K392R&lt;/sup&gt;</td>
<td>CUP1pr-aly2&lt;sup&gt;K392R&lt;/sup&gt; TRP1</td>
<td>2μ pRS426-ALY2pr-aly2&lt;sup&gt;K392R&lt;/sup&gt; (this study) was PCR amplified using primers that amplified the coding region of Aly2&lt;sup&gt;K392R&lt;/sup&gt; and contained XmaI and SalI restriction enzyme adaptors. This was cloned into pKK212 to create an N-terminal, in-frame GST fusion.</td>
</tr>
<tr>
<td>pRS415-TEF1pr-SEC61-FAP</td>
<td>TEF1pr-SEC61-FAP CEN LEU2</td>
<td>Primers containing BamHI and HindIII restriction site adaptors were designed to amplify the coding region of Sec61, without the stop codon. The PCR product was cloned into pRS415-TEF1pr plasmid (Mumberg et al., 1995) and verified by sequence analyses. The dL5 FAP tag (Yan et al., 2015) was then PCR amplified using primers containing HindIII and SalI restriction site adaptors and this product was cloned in frame downstream of Sec61. Plasmids were verified by sequence analyses.</td>
</tr>
<tr>
<td>pRS415-TEF1pr-SEC63-FAP</td>
<td>TEF1pr-SEC63-FAP CEN LEU2</td>
<td>Primers containing BamHI and HindIII restriction site adaptors were designed to amplify the coding region of Sec61, without the stop codon. The PCR product was cloned into pRS415-TEF1pr plasmid (Mumberg et al., 1995). The dL5 FAP tag (Yan et al., 2015) was then PCR amplified using primers containing HindIII and SalI restriction site adaptors and this product was cloned in frame downstream of Sec63. Plasmids were verified by sequence analyses.</td>
</tr>
<tr>
<td>pRS416-ZWF1pr-Kir2.1-HA</td>
<td>ZWF1pr-Kir2.1-HA CEN URA3</td>
<td>(Kolb et al., 2014)</td>
</tr>
</tbody>
</table>

### 2.2.3 Yeast protein extraction and immunoblot analysis

Whole-cell extracts of yeast proteins were made by growing cells to mid-exponential phase at 30°C in low-potassium SC medium supplemented with the indicated amount of KCl to mid-exponential phase at 30°C \((A_{600} = 0.6 – 0.8)\) and harvesting equivalent numbers of cells by
centrifugation. Cell pellets were flash-frozen in liquid nitrogen and stored at – 80°C until processing. Cells were then lysed using sodium hydroxide, and proteins were precipitated using trichloroacetic acid (TCA) as described (Volland et al., 1994b). The protein precipitates were solubilized in SDS/urea sample buffer (O'Donnell et al., 2013) and heated to 37°C for 15 min. Extracts were resolved by SDS-PAGE, and proteins were identified by immunoblotting with mouse anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) to detect Kir2.1 and rabbit anti-Zwf1 (glucose-6-phosphate dehydrogenase, referred to as G6PDH) antibody (Sigma) as a protein loading control. Anti-Sec61 antibody (Brodsky et al., 1993) and anti-Pma1 (40B7 Abcam, Cambridge, MA) were also used where indicated. Anti-mouse or anti-rabbit secondary antibodies conjugated to IRDye 800 or IRDye 680 (Li-Cor Biosciences, Lincoln, NE) were detected using an Odyssey™ Fc IR imaging system (LI-COR Biosciences), or where indicated, horseradish peroxidase-conjugated anti-rabbit immunoglobulin secondary antibody was applied (Jackson ImmunoResearch, West Grove, PA) and was detected with Supersignal chemiluminescent substrate (Pierce). In this case, images were processed on a BioRad ChemiDoc XRS+ image station with Image Lab 5.2.1 software (Hercules, CA).

2.2.4 Protein stability assays

Yeast cells lacking PDR5 and transformed with Kir2.1-HA or FAP-tagged Kir2.1 (expressed under the constitutive TEF1 promoter) were grown overnight to saturation, inoculated at an A600 of 0.25 into low-potassium SC medium supplemented with 100 mM KCl, and allowed to grow to mid-log phase at 30°C. To inhibit the proteasome, cultures were treated with 100 μM MG132 (Selleck Chemicals, Houston, TX) in DMSO or an equivalent amount of the DMSO
vehicle as a control, and incubated with agitation for 30 min at 37°C. At this point, 150 µg/ml cycloheximide (Sigma) was added to each culture to halt protein translation and cells were further incubated with agitation at 37°C. 1 ml aliquots were withdrawn at 0, 30, 60, and 90 min after the addition of cycloheximide, 17.5 mM NaN₃ was added, and cells were harvested by centrifugation and flash-frozen in liquid nitrogen. Whole-cell extracts were prepared by the TCA precipitation method described above and analyzed via SDS-PAGE and immunoblotting. Immunoblotting used the same antibodies and chemiluminescent methodology as described above for horseradish peroxidase conjugated antibodies.

2.2.5 Biochemical fractionation

The subcellular distribution of Kir2.1 was assessed by sucrose gradient sedimentation assays as described (Sullivan et al., 2003). In brief, 40 ml of the indicated yeast cultures were grown to an A₆₀₀ of 0.8, harvested by centrifugation, and lysed by glass-bead agitation as described (Kolb et al., 2014). Clarified cell lysates were layered onto a 30-70% sucrose gradient cushion and centrifuged at 100,000 X g in a Beckman SW41 rotor for 14 h at 4°C. Equal fractions were then collected by pipetting from the top of the tube, and proteins were assessed by SDS-PAGE and immunoblotting. Signal intensity was quantified by obtaining densitometry measurements of bands within the linear range of detection using ImageJ software (National Institutes of Health, Bethesda, MD).
2.2.6 Biochemical purification and MS analyses

GST-fused Aly1 or Aly2 was expressed from pKK212-derived plasmids under the control of the *CUP1* promoter by the addition of 200 μM CuSO₄ for 1 h. Protein extracts from these cells were generated by glass-bead lysis with vigorous agitation in co-immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 15 mM EGTA, 100 mM NaCl, 0.2% Triton X-100, 5 mM N-ethylmaleimide, and phosphatase inhibitors). GST fusion proteins were purified from equal concentrations of clarified lysates by incubation with GSH-Sepharose beads (GE Healthcare). The harvested proteins were washed in co-immunoprecipitation buffer and eluted in Laemmli buffer (Uetz et al., 2000). Proteins were then either resolved by SDS-PAGE and immunoblotted with α-ubiquitin and α-GST antibodies exactly as described (O'Donnell et al., 2013) to assess ubiquitination, or they were resolved by SDS-PAGE and stained with Coomassie Blue with the band corresponding to Aly1 or Aly2 excised for MS analyses. Proteins were excised from the gel and digested, and peptides were separated and analyzed by LC-MS/MS as described elsewhere (Tan et al., 2017). In brief, gel slices were destained with 50% acetonitrile and 25 mM ammonium bicarbonate before incubation with 10 mM DTT at 56°C for 1 h and a 1 h incubation with 55 mM iodoacetamide to reduce and alkylate proteins. Trypsin was added to the gel pieces, and proteins were digested overnight at 37°C. Proteolytic peptides were extracted with 70% acetonitrile and 5% formic acid, vacuum-dried, and reconstituted in 0.1% formic acid for LC-MS analyses. Peptides were analyzed by nano LC-MS/MS with a Dionex HPLC system (Dionex Ultimate 3000, Thermo Fisher Scientific) interfaced with a linear ion trap MS (LTQ-XL, Thermo Fisher Scientific). Peptides were separated on a C18 column (PicoChip column, New Objective, Inc., Woburn, MA). The mass spectrometer was run in data-dependent MS/MS mode so that each full
MS spectrum was followed by MS/MS scans of the eight most abundant molecular ions, and dynamic exclusion was enabled to reduce the selection of previously analyzed peptides for CID (collision-induced dissociation). MS/MS spectra were searched against the *Saccharomyces* Genome Database using the MASCOT search engine (version 2.4.0, Matrix Science Ltd.). Oxidation of methionine and carboxyaminomethylation of cysteines were set as variable and static modifications, respectively. Identification of the results were filtered with Scaffold (Proteome Software, Portland OR).

### 2.2.7 Inductively coupled plasma mass spectrometry

Total potassium levels in yeast cells were measured using inductively coupled plasma mass spectrometry (ICP-MS). Samples for these analyses were prepared essentially as described (Eide et al., 2005; Minear et al., 2011) with the following modifications. In brief, yeast cells containing the indicated plasmids were grown to saturation in low phosphate SC medium supplemented with 100 mM KCl. These cells were then washed in low phosphate SC medium with no KCl supplementation, inoculated at an $A_{600}$ of 0.2 into low-phosphate SC medium supplemented with 10 mM KCl, and grown for 6 – 8 h, which is when the cultures reached an approximate $A_{600}$ of 1.0. One $A_{600}$ unit’s worth of cells was then harvested by filtration on isopore membrane filters (Thermo Fisher) and washed twice in 1 μM EDTA and twice in double distilled water. The cells collected on the membrane filters were dissolved overnight in 1.5 ml of 30% nitric acid at 65°C. Samples were then diluted 15-fold to a final concentration of 2% nitric acid, and internal standards of beryllium, germanium, and thallium were added to all samples to evaluate signal strength and instrument drift. Sample metal concentrations were measured with a PerkinElmer NexION 300X
ICP-MS, calibrated with a 5-point calibration curve. A blank sample consisting of 2% nitric acid was run after every 7-10 samples to ensure that there were no signal memory effects. At least three biological replicates were run for each sample to ensure a robust set ICP-MS measurements.

2.2.8 FAP staining and confocal microscopy imaging

To assess FAP-Kir2.1 localization, yeast cells containing the pRS415-TEF1pr-FAP-TM-Kir2.1 plasmid, where the FAP corresponded to the dL5 version of this protein motif (Szent-Gyorgyi et al., 2013; Yan et al., 2015), were grown overnight to saturation in low-potassium SC medium supplemented with 100 mM KCl. The cells were then diluted to an $A_{600}$ of 0.2 in low-potassium SC medium with 100 mM KCl and grown for 4-5 h at 30°C until cells reached mid-exponential phase growth ($A_{600} = 0.5 – 0.7$). Where indicated, the cells were then treated with 200 µM LatA (Molecular Probes, Eugene, OR) or with an equal volume of DMSO vehicle for 2 h at 30°C prior to imaging. To allow the FAP tag to fluoresce, yeast cells were next incubated with 1 µM MG-B-TAU (cell-impermeant dye) or MG-ESTER (cell-permeant dye) for 15 min at room temperature and imaged. To this end, cells were plated onto 35-mm glass-bottom microwell dishes coated with poly-D-lysine (MatTek Corp., Ashland, MA) to ensure that the yeast remained stationary. Images were acquired using a Nikon Eclipse Ti inverted microscope outfitted with a Prairie-swept field confocal scan head, an Agilent monolithic laser launch, and an Andor iXon3 camera. NIS-Elements software was used to control the imaging parameters and all images within an experiment were captured using identical settings. Fluorescence of the FAP-bound MG-B-TAU and MG-ESTER dyes was excited using 636-nm light and the fluorescent emission was detected at 664 nm (Szent-Gyorgyi et al., 2013; Yan et al., 2015), which was achieved using the 640-nm
laser line. Maximum projections of 0.2 μm, Z-stacks through the entire cell were generated in ImageJ software (National Institutes of Health). All images within an experiment were adjusted equivalently, and an equal unsharp mask was applied to each image using Photoshop software (Adobe Systems Inc., San Jose, CA).

2.2.9 Image quantification and statistical analyses

The fluorescence intensity for all images was quantified using ImageJ software. To quantify total fluorescence with the MG-B-TAU cell-impermeant dye, maximum projections of 0.2μm Z-stacks through an entire cell were generated, and cells were outlined manually in ImageJ. Mean pixel intensities were measured in arbitrary units (a.u.) for each cell, and the mean background pixel intensity was subtracted. The distribution of mean pixel intensities in a.u. and the distribution of pixel intensities for each group of cells are presented as box-and-whisker plots, where the horizontal midline represents the median, the box is bounded by the upper and lower quartiles, and the whiskers denote maximal and minimal fluorescence intensities. A nonparametric Kruskal–Wallis test and Dunn’s multiple-comparison post hoc analyses were performed using Prism software (GraphPad, La Jolla, CA). Statistically significant differences are indicated in the figures by asterisks with the associated p values provided in the figure legends.
2.3 Results

2.3.1 A targeted screen identifies select α-arrestins as Kir2.1 regulators

In order for Kir2.1 to function, it must be trafficked to the PM. To assess the role the α-arrestins play in Kir2.1 trafficking we used a system developed to identify regulators of Kir2.1 surface activity (Kolb et al., 2014). In this yeast system the endogenous potassium channels, Trk1 and Trk2, are deleted and the ectopic expression of an exogenous potassium transporter is able to rescue growth on low-potassium medium (Kolb et al., 2014; Nakamura and Gaber, 1998; O'Donnell et al., 2017; Tang et al., 1995). Kir2.1 surface activity is measured using serial dilution assays on agar plates that are supplemented with high to low KCl. Cells lacking TRK1 and TRK2 with only a vector plasmid expressed are unable to grow on medium supplemented with low amounts of potassium. However, when Kir2.1 is expressed we see rescued growth on low potassium medium, 10 mM KCl, suggesting this yeast strain is able to use the mammalian potassium channel to import potassium into the cell and therefore raise intracellular potassium levels (Figure 4A, panel with pRS426 vector, A.F.O. and Figure 5A, N.A.H). To ensure the expression of Kir2.1 is rescuing growth due to the import of potassium, we assessed intracellular potassium levels using ICP-MS analyses on cells that has been growing for 6–8 h in low-potassium medium. Using ICP-MS, we are able to analyze the total intracellular element composition. We found that trk1Δ trk2Δ cells have ~40% less cellular potassium compared to WT, however, when we ectopically expressed Kir2.1 in trk1Δ trk2Δ cells potassium levels increased by ~20% (Figure 4B, A.F.O. and D.J.B.). As an additional negative control, we also expressed a mutant version of Kir2.1 which has the amino acids GYG mutated to alanine, Kir2.1-AAA (Kolb et al., 2014; Tinker et al., 1996). These mutations occur in the selectivity filter of Kir2.1, making it no longer
selective for potassium or functional. Kir2.1-AAA is unable to increase intracellular potassium levels compared to vector and is unable to rescue growth on low-potassium medium (Figure 4A with pRS426 vector, A.F.O. and B, A.F.O. and D.J.B.). This data supports the notion that ectopically expressing Kir2.1 in trk1Δtrk2Δ yeast directly increases intracellular potassium levels.

Previously this system had been used in a screen that identified many regulators of Kir2.1, however, due to the structure of the screen, which only looked at single-gene deletions, it would have missed genes that are functionally redundant to other genes. Therefore, we decided to complete a targeted screen where we selectively overexpressed a family of protein trafficking adaptors known as the α-arrestins. The α-arrestins are able to act in a functionally redundant manner where several of the α-arrestins are able to regulate a single membrane cargo, thus would remain undetected in the previous screen (Alvaro et al., 2014; Becuwe et al., 2012; Lin et al., 2008; Nikko and Pelham, 2009).

The α-arrestins aid in selective trafficking for many membrane cargo proteins including GPCRs, nutrient permeases and metal transporters (Alvaro et al., 2014; Becuwe et al., 2012; Lin et al., 2008; Nikko and Pelham, 2009; Nikko et al., 2008). Initial studies showed a role for α-arrestins in the endocytosis of cargo proteins, however, more recent studies reveal that they also play a role in intracellular sorting (Becuwe and Leon, 2014; Becuwe et al., 2012; Lin et al., 2008; Nikko and Pelham, 2009; O'Donnell et al., 2010; O'Donnell et al., 2015; Risinger and Kaiser, 2008). We hypothesize that this intracellular role of the α-arrestins is at play with Kir2.1.

To begin, 12 of the 14-known yeast α-arrestins were overexpressed in the trk1Δtrk2Δ background with either WT Kir2.1 or the negative control Kir2.1-AAA expressed (Figure 4A, A.F.O. and Figure 5B, A.F.O.) (Kolb et al., 2014; Tinker et al., 1996). Neither the 426-vector control nor the Kir2.1-AAA control are able to rescue growth on low potassium medium, however,
the α-arrestins are able to confer a range of rescue. α-Arrestins Rod1, Bul1, Ecm21, Csr2, and Art10 show no improvement over the vector control. Bul2, Rog3, Art5, and Rim8 show modest rescue. Three of the α-arrestins – Aly1, Aly2, and Ldb19 – are able to greatly improve growth on this low potassium medium in a Kir2.1-dependent manner (Figure 4A, A.F.O. and Figure 5B, A.F.O.). With this data in mind, we focused on these three α-arrestins and tested their ability to promote Kir2.1 localization to the PM.

Figure 4. α-Arrestins Aly1, Aly2, and Ldb19 promote Kir2.1-dependent growth on low-potassium medium
(A) Growth of serial dilutions of trk1Δ trk2Δ cells containing the indicated plasmids on SC medium lacking uracil and leucine and containing the indicated added concentrations of KCl is shown after 2 days. (B), WT (BY4741) or trk1Δ trk2Δ cells containing the indicated plasmids were grown in 10 mM KCl-containing medium, and then cellular potassium levels were measured using ICP-MS. The ppm of KCl for an equivalent number of cells was determined for four replicates, and the mean ± S.D. was calculated (presented as error bars). Pairwise Student’s t tests were performed to assess significance (***, p< 0.0002; ns, p>0.01, not significant) relative to trk1Δ trk2Δ cells with the vector control. (C) Lysates from cells expressing Kir2.1 and a vector control or overexpressing Aly1 were analyzed by sucrose gradient density fractionation (30-70%) under conditions to optimize separation of the ER and plasma membrane fractions. Fractions 1 and 14 are at the top and bottom of the gradient, respectively. Immunoblot analyses were used to detect Kir2.1 (α-HA antibody), ER-resident protein Sec61, and the plasma membrane protein Pma1. One of three representative experiments is shown. (D) the percentage of total Kir2.1 signal in the plasma membrane (fractions 8-14) was quantified using ImageJ for three replicate sucrose gradients; the mean percent signal ± S.D. are presented along with the individual data points for each replicate. A student’s t test was performed to assess significance (*, p <0.01).

Since each of these α-arrestins rescue growth on low potassium medium, we next wanted to determine if there was more Kir2.1 at the cell surface. However, we knew from previous work using indirect immunofluorescence and biochemical fractionation experiments that a majority of Kir2.1 is retained in the ER in this yeast model system and only a fraction of the population reaches the PM (Kolb et al., 2014). This suggests our screen on low potassium medium is sensitive. Additionally, we fractionated Kir2.1-expressing trk1Δ trk2Δ cells that had either a 426-vector control or 426-Aly1 overexpressed to assess any protein localization changes with the overexpression of this α-arrestin. Using sucrose gradient density centrifugation, we were able to resolve both ER and PM enriched fractions and examine Kir2.1 location (Figure 4C, P.G.N.) and see that, in agreement with previous work, a majority of Kir2.1 co-migrated with our Sec61-marked fractions which corresponded to the ER (Figure 4C, fractions 1-6, P.G.N.), whereas only ~12% migrated with our Pma1-marker for the PM fractions (Figure 4C, fractions 8-14, P.G.N.).
Furthermore, when we look at the extracts that have *ALY1* overexpressed, we can see the amount of *Kir2.1* that co-migrates with Pma1 (8-14) increased significantly (Figure 4, C and D, P.G.N.), yet the overall abundance of *Kir2.1* remains constant (Figure 5C, C.J.K.). Thus far, this data supports a role for these α-arrestins in the intracellular trafficking of *Kir2.1* where they promote *Kir2.1* trafficking to the PM.

Figure 5. *Kir2.1* rescues the growth of *trk1Δ trk2Δ* yeast on low potassium medium
(A) Growth of serial dilution of \textit{trk1}\Delta\textit{trk2}\Delta cells containing either a vector control or the pRS415-TEF1pr-Kir2.1 expression plasmid on SC medium lacking leucine and containing the indicated added concentration of KCl is shown. Please note that 0 mM KCl indicates that no additional KCl was added to the growth medium, however some residual potassium from the agar or in YNB powder is present. Growth shown is at 2 days. (B) Growth of serial dilutions of \textit{trk1}\Delta\textit{trk2}\Delta cells containing the indicated plasmids on SC medium lacking uracil and leucine and containing the indicated added concentrations of KCl is shown after 2 days of incubation. (C) Immunoblot of whole cell extracts from cells expressing ZWF1pr-Kir2.1-HA (detected with HA antibody) and over-expressing the indicated \(\alpha\)-arrestins. One of three representative blots is shown. Molecular masses are denoted in kilodaltons. Quantification of Kir2.1-HA abundance based on the immunoblot data is shown where error bars represent the SD and a Students \(t\)-test was used to assess significance relative to the vector (ns, p value > 0.01).

2.3.2 FAP-tagged Kir2.1 measures surface channel abundance

To better assess the ability of the \(\alpha\)-arrestins to promote Kir2.1 localization to the PM, we decided to utilize a recently developed imaging technique known as fluorogen activating proteins (FAPs). We know from previous work and ours that a majority of Kir2.1 resides in the ER, and this becomes problematic as the cortical ER runs just below the surface of the PM. Thus, we needed a way to distinguish between the two populations. Using traditional imaging techniques this would be challenging, however, using FAPs makes it manageable. This alternative strategy makes use of fluorogen activating proteins. There are two components of FAPs. There is the malachite green (MG)-derived dye and a single chain antibody (SCA) that is fused to the protein of interest (Szent-Gyorgyi et al., 2008) (Fig. 6A, A.F.O.). When these components are separated there is no fluorescence, but when they bind together there is a 20,000-fold increase in fluorescence (Szent-Gyorgyi et al., 2013; Yan et al., 2015). What makes this technique of specific interest to us, is the different MG-derived dyes that can be used. There is an MG-ESTER dye that is cell permeant,
which shows the total cellular pool of the protein of interest, and there is a cell-impermeant, MG-B-TAU, that is unable to cross the plasma membrane due to a polar side chain allowing visualization of the protein only out at the PM (Figure 6B, A.F.O.). The cell-impermeant dye, MG-B-TAU, is of particular interest to us because we only want to visualize the pool of Kir2.1 at the PM.

**Figure 6. The use of a fluorgen-activating protein to report on cellular residence**

(A) The FAP technique makes use of a MG-derived dye that binds an SCA. Neither the MG nor the SCA is fluorescent, but when the MG dye is bound by a SCA, fluorescence is detected (B) The MG-derived dye can be conjugated to a membrane soluble side chain (MG-ESTER, cell-permeant dye) that freely passes through the yeast cell wall and the plasma membrane, where is can be bound by intracellular SCAs to activate fluorescence and monitor the intracellular levels of a tagged protein. The MG-derived dye can also be conjugated to a membrane-impermeant side chain, as is the case with the MG-B-TAU dye. This dye can no longer enter the cell, so only the SCAs on the external face of the cell surface will be bound by dye and fluoresce.

In order to use the cell-impermeant dye, the SCA must be on the extracellular side of the PM, however, both the N- and C-termini of Kir2.1 are in the cytosol (Hibino et al., 2010). To
overcome this, we attached an N-terminal transmembrane domain to Kir2.1 and then added the FAP sequence upstream of this newly inserted transmembrane domain (Figure 6A, A.F.O.). This construct was under control of the *TEF1* promoter, which is a strong constitutive promoter.

To confirm that MG-B-TAU was cell-impermeant in yeast, we generated two plasmids where two ER resident markers, Sec61 and Sec63, were tagged with the FAP SCA under the *TEF1* promoter. Sec61 and Sec63 are stably expressed in the ER as they are both part of the protein translocation machinery in the yeast ER (Corsi and Schekman, 1996). Each of these plasmids, along with the FAP-TM-Kir2.1, were transformed into *trk1Δ trk2Δ* cells and imaged using confocal microscopy after being incubated with either the cell-permeant (MG-ESTER) or cell-impermeant (MG-B-TAU) dyes for 30 min. When MG-ESTER dye was used, Sec61-FAP and Sec63-FAP localize nicely with the ER, displaying the perinuclear and cortical ER rings (Preuss et al., 1991). Whereas, when FAP-TM-Kir2.1 was incubated with MG-ESTER, it had a punctate pattern and displays patches throughout the cortical ER (Fig. 7B, top row, N.A.H.). Using the MG-ESTER, it was impossible to distinguish between the pool of FAP-TM-Kir2.1 at the PM and in the ER. However, when we used the MG-B-TAU dye and imaged using the same parameters, we were no longer able to visualize Sec63-FAP, but there was a fluorescence signal for FAP-TM-Kir2.1 that can more clearly be seen when images were adjusted using post-acquisition modifications unlike with Sec63-FAP (Fig. 7B, second and third rows, N.A.H.). The FAP-TM-Kir2.1 fluorescence intensity is modest; however, this aligns well with only a small pool (~10%) of Kir2.1 localizing to the PM and is significantly brighter than our Sec63-FAP control when we quantify these respective cells (Figure 4 C and D, P.G.N.; Figure 7D, N.A.H.). Additionally, when we quantify both the MG-ESTER and the MG-B-TAU exposed cells the MG-ESTER incubated cells are brighter than MG-B-TAU dye (Figure 7, C and D, N.A.H.). Furthermore, for both FAP-TM-Kir2.1
and Sec63-FAP the more time incubated in the dye the brighter the signal. Thus, indicating with longer incubation times, some of the cell-impermeant dye may enter the cells over time resulting from bulk endocytosis (Figure 7D, N.A.H.).

Figure 7. FAP-tagged Kir2.1 can be detected at the cell surface
(A) Schematic of FAP-tagged Kir2.1. Kir2.1 transmembrane domains are shown in blue, and the platelet-derived growth factor transmembrane domain is shown in green. The FAP tag extends from the N terminus and is shown as gray ovals. (B) Cells expressing FAP-TM-Kir2.1, Sec63-FAP, or Sec61-FAP were incubated with the MG-ESTER or MG-TAU dye to activate intracellular or cell-surface FAP-tag fluorescence, respectively. Confocal microscopy images of medial sections of cells incubated with dye for 30 min are shown. In the top two rows, cells are adjusted equivalently to show that the signal from intracellular FAP-TM-Kir2.1 or Sec63-FAP is lost when the MG-B-TAU dye is used. Yellow dashed-line circles are used in the second row to mark the cell. The bottom row of images are the same images shown in the second row but are adjusted to allow the cell-surface fluorescence of the MG-B-TAU-stained FAP-TM-Kir2.1-expressing cells to be seen. (C and D) Total cellular fluorescence for n > 70 cells, imaged as in B, was measured, and mean fluorescence intensities for all cells (in a.u.) are presented as scatter plots. The horizontal midline in each plot represents the mean fluorescence intensity, and the error bars represent ± S.D. (D) The cellular fluorescence was quantified at both 15- and 30-min post-MG-B-TAU addition. Kruskal-Wallis statistical analyses with Dunn’s post hoc test were performed, and the statistical significance is indicated (**, p<0.001; ***, p<0.0001; ns, p>0.01, not significant). (E) Confocal microscope images of cells expressing FAP-TM-Kir2.1 that were incubated for 90 min with LatA or DMSO and stained with MG-B-TAU for 30 min are shown. (F) The total cellular fluorescence for n > 60 cells, imaged as in E, was measured, and mean fluorescence intensities for all cells (in a.u.) are presented as scatter plots. A Student’s t test was performed to assess significance (***, p <0.0001).

To confirm that the punctate seen at the cell periphery were indeed the PM pool of FAP-TM-Kir2.1, we pretreated cells with latrunculin A (LatA) (Figure 7, E and F, N.A.H.). LatA is able to halt endocytosis, but leave intracellular sorting alone, by blocking actin polymerization (Ayscough et al., 1997). Cells treated with LatA resulted in the accumulation of FAP-TM-Kir2.1 at the cell surface, which could be seen when we use the cell-impermeant dye, MG-B-TAU, and was quantifiable (Figure 7, E and F, N.A.H., A.V.K.). Thus far, this data establishes that MG-B-TAU dye is indeed cell-impermeant in yeast as it has been reported to be so in mammalian cells (Yan et al., 2015), and can be used to quantify the cell surface abundance of FAP-TM-Kir2.1.
To ensure functionality and stability of the newly generally FAP-TM-Kir2.1 construct, we conducted serial dilution assays as described above and saw that similarly to our WT Kir2.1, this construct also improved growth on low-potassium medium compared to vector control. However, FAP-TM-Kir2.1 rescued growth less well than WT Kir2.1 (Figure 8A, A.F.O.). Unsurprisingly, steady-state levels of FAP-TM-Kir2.1 were dramatically lower than WT Kir2.1, as can be seen via immunoblot analysis of whole-cell extracts (Fig. 8B, C.J.K.). As both the WT Kir2.1 and FAP-TM-Kir2.1 are both expressed from the same promoter, this disparity is likely the result of their respective degradation rates. As expected, FAP-TM-Kir2.1 was significantly less stable than WT Kir2.1 as seen via cycloheximide chases (Figure 8, C and D, T.D.M.). Furthermore, when the proteasome was inhibited via incubation with MG132, there is significant stabilization of FAP-TM-Kir2.1(Figure 8, C and D, T.D.M.), which demonstrates it is degraded via the proteasome.

Since we are analyzing the trafficking of this construct, it was important to determine if the degradation of FAP-TM-Kir2.1 was regulated in the same way as was reported for WT Kir2.1, which showed regulation by retromer (Kolb et al., 2014). Specifically, Kolb et al. showed that when cells lacked the retromer subunit Vps35 (trk1Δ trk2Δ vps35Δ), Kir2.1-dependent growth on low-potassium medium was greatly improved compared to trk1Δ trk2Δ cells and we saw a similar trend with FAP-TM-Kir2.1 (Figure 8A, A.F.O.). Additionally, in trk1Δ trk2Δ vps35Δ cells, there were increased levels of Kir2.1 at the PM (Figure 8, E and F, A.F.O., A.V.K.). Altogether, this data implies that although FAP-TM-Kir2.1 is less stable than WT Kir2.1, it is still a function channel whose trafficking is equivalent to WT Kir2.1 (Kolb et al., 2014).
Figure 8. The plasma membrane residence and activity of Kir2.1 and FAP-tagged Kir2.1 are regulated similarly

(A) Growth of serial dilutions of trk1Δ trk2Δ or trk1Δ trk2Δ vps35Δ cells containing the indicated plasmids on SC medium lacking leucine and containing the indicated amount of KCl is shown. Growth shown is at 4 days. (B) Whole-cell extracts from trk1Δ trk2Δ cells expressing Kir2.1 of FAP-TM-Kir2.1 were analyzed by immunoblotting. Red dots indicate bands of the correct molecular mass for Kir2.1 and FAP-TM-Kir2.1 (α-HA). Molecular masses are denoted in kilodaltons. Whole-cell extracts were loaded at two different concentrations (lanes 1 and 2) as shown in the loading control (α-G6PDH). Two different exposures of the same blot are shown to facilitate comparisons. (C) Yeast cells (trk1Δ trk2Δ pdr5Δ) expressing either HA-tagged or FAP-tagged Kir2.1 were grown to mid-logarithmic phase, treated with either MG132 or DMSO (vehicle control), and dosed with cycloheximide (CHX), and samples were taken at the indicated time points. Kir2.1 protein abundance was assessed by immunoblotting with the indicated antibodies. Molecular masses are denoted in kilodaltons. (D) Immunoblots as in C were quantified for a minimum of
four replicate experiments, and the means are plotted. Data are normalized to 100% for $t = 0$ time point in each case. Error bars represent ± S.D., and a Student’s $t$ test was performed to assess significance (**, $p < 0.001$; ***, $p < 0.0001$; ns = $p > 0.01$, not significant). (E) $trk1\Delta trk2\Delta$ or $trk1\Delta trk2\Delta vps35\Delta$ cells expressing FAP-TM-Kir2.1 were treated with LatA or DMSO for 90 min and then incubated with MG-B-TAU dye for 30 min and imaged by confocal microscopy. (F) Total cellular fluorescence for $n > 50$ cells, imaged as in C, was measured, and mean fluorescence intensities for all cells (in a.u.) is presented as scatter plots. A Student’s $t$ test was performed to assess significance (***, $p < 0.001$).

### 2.3.3 α-Arrestins increase Kir2.1 residence at the plasma membrane in a Rsp5-dependent manner

Select α-arrestins were able to rescue growth on low potassium medium and increase intracellular potassium levels, thus we hypothesized that the overexpression of the α-arrestins would increase Kir2.1 levels at the PM. In order to test this, we utilized our FAP-TM-Kir2.1 construct. When we overexpressed α-arrestins Aly1, Aly2, and Ldb19 and imaged using MG-B-TAU we saw increased fluorescence indicating increased levels of FAP-TM-Kir2.1 at the PM compared to vector (Figure 9, A and B, A.F.O.). We then treated cells with LatA, which inhibits endocytosis in yeast yet leaves intracellular trafficking unaffected (Ayscough et al., 1997; Coue et al., 1987; Kaksonen et al., 2003), to confirm that the α-arrestins were involved in the delivery of FAP-TM-Kir2.1 to the PM and not in endocytosis. With the addition of LatA, we observed increased levels of FAP-TM-Kir2.1 cell surface fluorescence when Aly1, Aly2, and Ldb19 were overexpressed compared to vector control (Figure 9, C and D, N.A.H.), demonstrating that α-arrestins promote FAP-TM-Kir2.1 localization to the PM independently of endocytosis.
Figure 9. α-Arrestin regulation of Kir2.1 requires the Rsp5 ubiquitin ligase

(A) Maximum Z-projections of confocal microscopy images were acquired in trk1Δ trk2Δ cells expressing FAP-TM-Kir2.1 and in the presence of a vector control or plasmids overexpressing the indicated α-arrestins. The cells were incubated with MG-B-Tau dye for 30 min. (B) Total cellular fluorescence for the maximum Z-projections of n>30 cells, imaged as in A, was measured, and mean fluorescence intensities for all cells (in a.u.) are presented as scatter plots. The horizontal midline in each plot represents the mean fluorescence intensity, and the error bars represent ± S.D. Kruskal-Wallis statistical analyses with Dunn’s post hoc test were performed, and the statistical significance compared with the vector control is indicated (p values are assigned as listed below). (C) Maximum Z-projections of confocal microscopy images were acquired in trk1Δ trk2Δ cells expressing FAP-TM-Kir2.1 and containing the vector control or plasmids overexpressing the indicated α-arrestins. The cells were incubated with LatA (200 µM), for 90 min and the MG-B-TAU dye for 30 min. (D) Total cellular fluorescence for the maximum Z-projections of n>50 cells, imaged as in C, was measured, and mean fluorescence intensities for all cells (in a.u.) are presented as scatter plots. The horizontal midline in each plot represents the mean fluorescence intensity, and the error bars represent ± S.D. Kruskal-Wallis statistical analyses with Dunn’s post hoc test were performed (*, p < 0.01; **, p < 0.001; ***, p < 0.0001; ****, p < 0.00001; ns, p > 0.01, not significant).
As previously mentioned in Chapter 1, all yeast α-arrestins contain at least one $L/P_{XX}Y$ motif that binds the WW-domains of the ubiquitin ligase Rsp5. Rsp5 binding is required for α-arrestin-dependent trafficking of cargo proteins (Hatakeyama et al., 2010; Lin et al., 2008; Nikko and Pelham, 2009; O'Donnell et al., 2015; Rotin et al., 2000). To assess if Kir2.1 trafficking is dependent on α-arrestin’s ability to bind of Rsp5, the $L/P_{XX}Y$ motifs were mutated. Alvaro et al. mutated this motif to PAXA in each of these α-arrestins, and for Rog3 the VPXY motif was mutated as well. Each of these mutants dramatically decreased the amount of Rsp5 that copurified with each GST-tagged α-arrestin. They found that although Ldb19 and Rod1 down regulate Ste2 through recruitment of Rsp5 via their $L/P_{XX}Y$ motifs, Rog3 is able to promote Ste2 down regulation with only the arrestin fold domain thus it does not require the $L/P_{XX}Y$ motif (Alvaro et al., 2014).

To assess the dependence of Rsp5 on this α-arrestin-mediated trafficking of Kir2.1 we employed $L/P_{XX}Y$ mutants ($L/P_{XG}$, referred to as Aly1PPXY-less or PPXY-less mutants) for Aly1, Aly2, and Ldb19. First we took our PPXY-less mutants for Aly1 and Aly2 and tested their sensitivity to azetidine-2-carboxylic acid (AzC), a toxic analog of proline. AzC is able to enter the cell via Gap1, which has been shown to be regulated by the α-arrestins. When the PPXY-less mutants are overexpressed, cell sensitivity to AzC was lost, implying there was a defect in Gap1 trafficking to the PM (Figure 10C, A.F.O) (O'Donnell et al., 2010; O'Donnell et al., 2013). When the ability of these mutants to rescue growth on low-potassium medium was tested, they were dramatically less effective compared to their WT counterparts (Figure 11A, A.F.O.). Additionally, these mutants were not able to increase Kir2.1 levels at the PM to the degree of WT (Figure 9, A.F.O.). Specifically, Aly1 and Ldb19 did not significantly increase Kir2.1 levels at the PM compared to vector, but there was a modest increase with Aly2$^{PPXY}$-less (Figure 9 A and B,
A.F.O.). Consistent with these findings, the PPXY-less mutants were unable to elevate intracellular potassium levels (Figure 11B, N.A.H.). Altogether, this data gives support that these select α-arrestin must bind Rsp5 to promote Kir2.1 trafficking to the cell surface.

**Figure 10. Aly1 and Aly2 are ubiquitinated at K379 and K92**

(A and B) The amino acid sequences of (A) Aly1 and (B) Aly2 with numbers on the left indicating amino acid position is presented. Protein sequences highlighted in grey indicate where mass spectroscopy corresponding to peptides identified in our analyses. The precent coverage is indicated above the chart along with the total number of...
spectra mapper to either Aly1 or Aly2. Regions absent from the mass spectroscopy analysis generally lack trypsin cleavage sites or contain numerous trypsin cleavage sites, which likely generate peptides that are too long or too short, respectively, to be defined. Lysines highlighted in yellow and bold text were identified as having the diglycine mass shift associated with ubiquitination. Lysines in bold and underlined were previously identified as being modified in other ubiquitin proteomics studies. (C) Growth of serial dilutions of BY4743 cells containing the indicated pRS426-based plasmids on SC medium lacking uracil (SC) or minimal medium containing only the amino acids required for survival (MIN) and either 500 ng/ml rapamycin or 50 µg/ml azeditidine-2-carboxylic acid (AZC). Cell growth at 4-5 days is shown.

**Figure 11.** Overexpression of Aly1, Aly2, and Ldb19 increases both Kir2.1 residence at the plasma membrane and intracellular potassium

(A) Growth of serial dilutions of trk1Δ trk2Δ cells containing the indicated plasmids on SC medium lacking leucine and uracil and containing the indicated added amounts of KCl is shown after 2 days. (B) trk1Δ trk2Δ cells expressing Kir2.1 and the indicated overexpressed α-arrestin plasmids were grown in 10 mM KCl, and cellular

68
potassium levels were measured using ICP-MS. The ppm corresponding to the KCl for an equivalent number of cells was determined for four replicates, and the mean ± S.D. was calculated (presented as error bars). Kruskal-Wallis statistical analyses with Dunn’s post hoc test were performed, and the statistical significance compared with the vector control for each is indicated (**, p < 0.001; ***, p < 0.0001; ns, p > 0.01, not significant). (C) Schematic of the Aly1 and Aly2 protein coding regions, where the numbers indicate the amino acid position, dark red boxes denote the N-terminal arrestin-fold, the light red boxes denote the C-terminal arrestin-fold, green ovals indicate the position of the (L/P)PXY motifs, and blue circles indicate the sites of ubiquitin (Ub) modification identified via MS. (D) GST-fused 𝛼-arrestins were isolated from WT (BJ5459) cells after 𝛼-arrestin expression (under control of the CUP1 promoter) was induced with copper sulfate. Isolated proteins were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. Molecular masses are denoted in kilodaltons. (E) Growth serial dilutions of trk1∆ trk2∆ cells containing the indicated plasmids on SC medium lacking leucine and uracil and containing the indicated added amounts of KCl is shown after 4 days.

Next, we wanted to better understand the relationship between Rsp5 and the 𝛼-arrestins. For example, the 𝛼-arrestins can be monoubiquitinated by Rsp5 and perhaps this plays a role in the ability of the 𝛼-arrestins to traffic Kir2.1. Thus far, loss of monoubiquitination of the 𝛼-arrestins has been linked to impairment of the 𝛼-arrestins ability to traffic to the PM and interact with other trafficking factors (Becuwe et al., 2012; Herrador et al., 2013; Lin et al., 2008). To analyze the effect this would have on the 𝛼-arrestin-mediated trafficking of Kir2.1 with Aly1 and Aly2, we used LC-MS analysis to assess GST-tagged Aly1 and Aly2 from WT yeast. We were able to obtain spectra corresponding to peptides covering 60 (Aly1) and 56% (Aly2) (Figure 10, A and B, A.F.O., A.A.A.). From this di-glycine-modified lysines, which indicated ubiquitination in MS spectra (Kim et al., 2011; Udeshi et al., 2012; Xu et al., 2009), were identified at the positions labeled onto the Phyre-predicted secondary structures of Aly1 and Aly2 (Figure 11C, A.F.O. and Figure 10, A and B, A.F.O. and A.A.A.) (Bennett-Lovsey et al., 2008; Kelley and Sternberg, 2009; O'Donnell et al., 2010). It is important to note that our data is consistent with that
of previous literature, as Lys-392 in Aly2 was previously defined in other proteomic studies (Starita et al., 2012; Swaney et al., 2013; Ziv et al., 2011) as well as Lys-379 in Aly1 (Swaney et al., 2013). With this data in mind, we mutated Lys-392 in Aly2 and the analogous Lys-379 residue in Aly1 to arginine using site-directed mutagenesis. Using immunoblot analysis, we could see this mutation resulted in the loss of monoubiquitination as Aly1^K379R and Aly2^K392R lacked the ubiquitin signal that could be easily detected with WT Aly1 and Aly2 (Figure 11D, A.A.A.). Consistent with the notion that this ubiquitination results from Rsp5, we used our PPXY-less mutants and again saw a lack of ubiquitin signal (Figure 11D, A.A.A.) as we have seen previously (O'Donnell et al., 2013). Together, these results confirm that these lysines are indeed ubiquitinated by Rsp5.

In order to fully understand the impact that Lys-to-Arg mutations would impose on Aly1 and Aly2, we conducted a series of experiments to compare the phenotype of these mutants to those of their WT counterparts. First, it is known that overexpression of WT Aly1 and Aly2 confers resistance to cells when plated on medium with rapamycin (O'Donnell et al., 2010; O'Donnell et al., 2013). We found that when Aly1 and Aly2 were unable to be ubiquitinated Aly1 still conferred rapamycin resistance, while Aly2 was unable to confer resistance (Figure 10C, A.F.O., A.A.A.). Next, we analyzed their ability to confer sensitivity to AzC. Both Aly1^K379R and Aly2^K392R were less effective at conferring this sensitivity, indicating both of these α-arrestins ability to promote Gap1 trafficking to the PM is reduced (Figure 10C, A.F.O., A.A.A.). Together, this data demonstrates that ubiquitination is important for some functions linked to these two α-arrestins, but not all of them.

Now that we had assessed the affect ubiquitination has on these α-arrestins with other cargo, we turned focus back to Kir2.1. Both \textit{ALY1}^{K392R} and \textit{ALY2}^{K379R} and their PPXY-less
counterparts had diminished capacity to rescue growth on low-potassium medium when compared to their WT counterparts (Figure 11E, N.A.H.). This data implies that the ubiquitination of Aly1 and Aly2 by Rsp5 is needed for optimal α-arrestin-mediated trafficking of Kir2.1 to the PM.

2.3.4 α-Arrestins phosphorylation impacts Kir2.1 trafficking

As mentioned previously, α-arrestins have been shown to work in two distinct pathways. The traditional endocytosis pathway and in intracellular sorting. This switch has been hypothesized to be dependent on the phosphorylation status of the α-arrestin. It is unsurprising that phosphorylation would play a role in α-arrestin regulation as they are known to be heavily phosphorylated. For example, there have been 24 and 13 phosphorylated residues identified in Aly1 and Ldb19, respectively using MS (Albuquerque et al., 2008; Holt et al., 2009; MacGurn et al., 2011; O'Donnell et al., 2013; Swaney et al., 2013). In most cases, phosphorylation of the α-arrestins has hindered their ability to stimulate endocytosis of their respective cargo protein, whereas dephosphorylated α-arrestins strengthen their ability to promote endocytosis (Becuwe et al., 2012; Crapeau et al., 2014; MacGurn et al., 2011; Merhi and Andre, 2012; O'Donnell et al., 2013). For example, when Ldb19 (Art1) is phosphorylated by the protein kinase Npr1, it is unable to promote the endocytosis of the arginine transporter, Can1 (MacGurn et al., 2011). Consistent with this notion is work from our lab that focuses on the phosphorylation state of Aly1. Work from our lab identified Aly1 as a substrate of the phosphatase calcineurin, which is a calcium and calmodulin-dependent phosphoprotein phosphatase that regulates a wide range of processes in mammals (Aramburu et al., 2004; O'Donnell et al., 2013). Specifically, the dephosphorylation of Aly1 by calcineurin is essential for its ability to internalize the aspartic acid/glutamic acid
permease Dip5 (O’Donnell et al., 2013). The interaction interface between Aly1 and calcineurin was mapped using a yeast two-hybrid analysis between the catalytic subunit of calcineurin, Cna1, and mutants of Aly1. These experiments identified a consensus PXIXIT-docking motif, with the amino acid sequence PILKIN in Aly1 as required for the interaction with and dephosphorylation of Aly1 (O’Donnell et al., 2013). When this motif was mutated to alanines and thus Aly1 was unable to be dephosphorylated by calcineurin, the Aly1-mediated endocytosis of Dip5 was inhibited (O’Donnell et al., 2013). Interestingly, the inability of Aly1 to be dephosphorylated did not affect its ability to recycle the general amino acid permease, Gap1 (O’Donnell et al., 2013).

Given that we hypothesize that α-arrestins are involved with the intracellular sorting of Kir2.1, we decided to assess the effect its phosphorylation status would have on its ability to traffic Kir2.1. With a mutated version of Aly1, where the PILKIN (aa 832-837) motif was mutated to alanines (ALY1AAAAAA) (O’Donnell et al., 2013), we first overexpressed this protein and analyzed its ability to rescue growth on low-potassium medium (Figure 12, A.F.O.). Aly1AAAAAA further rescued growth compared to its WT counterpart, suggesting that constitutive phosphorylation enhanced Kir2.1 trafficking to the PM. In line with this notion, Aly1AAAAAA overexpression increased FAP-TM-Kir2.1 levels at the PM (Figure 12, B-C, N.A.H.) and increased intracellular potassium levels as assessed via ICP-MS (Figure 12D, N.A.H., D.J.B.). Furthermore, to ensure the effect was due to the calcineurin interaction with Aly1, we deleted a calcineurin regulatory subunit, CNB1, that is required for catalytic activity (Cyert and Thorner, 1992), in the trk1Δ trk2Δ strain background. In this strain we then tested the ability of WT Aly1 to promote Kir2.1-dependent growth on low-potassium medium and found that without calcineurin activity the ability of Aly1 to rescue growth improved (Figure 12E, N.A.H.). Together, these data support a model that calcineurin dephosphorylation of Aly1 inhibits its ability to promote Kir2.1 trafficking to the PM (Figure 9F,
A.F.O.). Taken together with previous work in the field this data supports the notion that the phosphorylation status of the α-arrestin, Aly1, is able to act as a switch wherein dephosphorylation stimulates endocytosis, while phosphorylated α-arrestins participate in intracellular trafficking of select integral membrane proteins and promote their trafficking to the PM (Figure 12F, A.F.O).

**Figure 12. Calcineurin negatively regulates Aly1-mediated Kir2.1 trafficking to the plasma membrane**

(A) Growth of serial dilutions of trk1Δ trk2Δ cells containing the indicated plasmids on SC medium lacking leucine and uracil and containing indicated amounts of KCl is shown after 2 days. (B) Maximum Z-projections (Max Z) of confocal microscopy images acquired in trk1Δ trk2Δ cells expressing FAP-TM-Kir2.1 and containing a vector control or plasmids overexpressing the indicated α-arrestins. Cells were incubated with MG-B-TAU dye for 30 min. (C) Total cellular fluorescence for the maximum Z-projections of n> 45 cells, imaged as in B, was measured, and mean fluorescence intensities for all cells (in a.u.) are presented as scatter plots. The horizontal midline in each plot
represents the mean fluorescence intensity, and the error bars represent ± S.D. Kruskal-Wallis statistical analyses with Dunn’s post hoc test were performed, and the statistical significance compared with the vector control is indicated.

(D) trk1Δ trk2Δ cells expressing Kir2.1 and the indicated overexpression α-arrestins plasmids were grown in 10 mM KCl and cellular potassium levels were measured by ICP-MS. The PPM of KCl for an equivalent number of cells was determined for 4 replicates, and the mean and S.D. were calculated (presented as error bars). Kruskal-Wallis statistical analyses with Dunn’s post hoc test were performed, and the statistical significance compared with the vector control for each is indicated (*, p < 0.01; **, p < 0.001; ***, p < 0.0001; ns, p > 0.01, not significant). (E) Growth of serial dilutions of trk1Δ trk2Δ or trk1Δ trk2Δ cnb1Δ cells containing the indicated plasmids on SC medium lacking leucine and uracil and containing the indicated amount of KCl is shown after 2 days. (F) Model for calcineurin (CN) regulation of Aly1-mediated trafficking of Kir2.1. Red dashed lines indicate exocytosis or endocytosis of Kir2.1. Gray circles on Aly1 indicate phosphorylation sites, with fewer phosphorylation sites in the version of Aly1 that has been dephosphorylated by calcineurin.

2.4 Discussion

In this Chapter, we were able to employ a yeast genetic screen to identify a family of protein trafficking adaptors as regulators of the mammalian potassium channel Kir2.1. Specifically, three select α-arrestins – Aly1, Aly2, and Ldb19 – were shown to promote Kir2.1 trafficking to the plasma membrane and increase intracellular potassium levels. To support this notion, we completed a series of experiments: 1) we showed that the overexpression of Aly1, Aly2, and Ldb19 rescued growth on low potassium medium in a Kir2.1-dependent manner via serial dilution assays, 2) using ICP-MS, we were able to analyze the composition of yeast cells and verified overexpression of these α-arrestins increased intracellular potassium levels thus confirming these select α-arrestins directly alter Kir2.1 function at the plasma membrane, and 3) for the first time in yeast, we utilized the fluorescent activating protein technique to quantify plasma membrane
localized FAP-tagged Kir2.1. Furthermore, this work confirmed previous studies that shed light on how the α-arrestins are themselves regulated specifically with their phosphorylation status and their ability to interact with the ubiquitin ligase, Rsp5.

Due to the complexities of working with the primary cardiac myocytes in which Kir2.1 resides, it is extremely advantageous to use yeast as a model system for studying Kir2.1 trafficking. Since its discovery as a model system for studying potassium channels in the 1990s, it has aided in various studies as outlined in Mackie et al. (Mackie and Brodsky, 2018; Nakamura and Gaber, 1998). Our work in this system was sparked by the work of Kolb et al. Specifically, over 60% of the Kir2.1 regulators they identified were trafficking factors. Furthermore, they were able to provide evidence that their results in yeast were able to be confirmed in mammalian cells thus creating confidence in this system. Using this model system they found that members of the endosomal sorting complex required for transport (ESCRT) were responsible for Kir2.1 degradation, which was then found to be true in HeLa cells as well (Kolb et al., 2014).

Based on our findings, we suspect the α-arrestin-mediated trafficking of Kir2.1 is part of the recycling pathway by promoting Kir2.1 localization to the plasma membrane. However, it is important to note that this is a relatively new role for the α-arrestins to function in as a majority of studies focus on their ability to stimulate endocytosis (Becuwe et al., 2012; Hatakeyama et al., 2010; Lin et al., 2008; Nikko and Pelham, 2009; Nikko et al., 2008; O'Donnell et al., 2015). Interestingly, α-arrestins Aly1 and Aly2 are linked with intracellular sorting. For example, Aly1 and Aly2 promote the trafficking of the general amino acid permease, Gap1, to the cell surface in response to nutrient depletion (O'Donnell et al., 2010). Consistent with our results, the α-arrestin-mediated trafficking of Gap1 was also dependent on regulation of the α-arrestin itself via its phosphorylation and ubiquitination status. In addition, Aly2-mediated trafficking of Gap1 is
dependent on its phosphorylation by the kinase Npr1. Npr1 is known to be negatively regulated by TORC1 (Target of Rapamycin Complex), while it is a positive regulator for Gap1 trafficking to the plasma membrane (Boeckstaens et al., 2014; De Craene et al., 2001; MacGurn et al., 2011; O'Donnell, 2012; O'Donnell et al., 2010). Additionally, in order to traffic Gap1, Aly2 must interact with the clathrin adaptor complex, AP-1. AP-1 is responsible for endosome-to-Golgi retrieval of select membrane cargo (Foote and Nothwehr, 2006; Valdivia et al., 2002; Valdivia and Schekman, 2003). This previous data from our lab supports the model where Aly2 promotes Gap1 trafficking from the endosomes to the trans-Golgi network via AP-1 and clathrin-coated vesicles. Furthermore, Aly2 is regulated by Npr1 and must be phosphorylated by Npr1 in order to initiate Aly2-mediated recycling of Gap1 (O'Donnell et al., 2010). We hypothesize that Aly2 uses a similar pathway when promoting Kir2.1 trafficking to the cell surface.

In order to fully depict how the α-arrestins are regulated via phosphorylation, we assessed the effect calcineurin-associated Aly1 dephosphorylation had on Kir2.1 trafficking to the cell surface. This dephosphorylation by calcineurin inhibited the ability of Aly1 to promote Kir2.1 trafficking to the cell surface. This is significant, as previous work from our lab and others has found that dephosphorylation of Aly1 stimulates endocytosis (Hatakeyama et al., 2010; O'Donnell et al., 2013). Specifically, Aly1 must interact with and be dephosphorylated by calcineurin in order to decrease cell surface levels of Dip5, an aspartic acid/glutamic acid permease (O'Donnell et al., 2013). Together, this data supports a model in which phosphorylation/dephosphorylation of the α-arrestins act as a switch between the role of recycling and endocytosis. To further support this model, calcineurin has been shown to negatively regulate Kir2.1 in more complex systems. Using mammalian cells (COS7), Dart and Leyland have shown Kir2.1 interacts with the A kinase-anchoring proteins (AKAP), AKAP79. AKAP79 is a scaffolding protein that harbors calcineurin
among other kinases and phosphatases, and this interaction increases Kir2.1 channel function when phosphatases are inhibited via okadaic acid and cypermethrin (Dart and Leyland, 2001). This supports the hypothesis that dephosphorylation is a way to negatively regulate Kir2.1 activity at the cell surface. Furthermore, in *Xenopus oocytes*, inhibition of calcineurin via the immunosuppressant cyclosporin A decreases Kir2.1 channel activity at the cell surface (Chen et al., 1998).

Excitingly, in contrast to Aly1 and Aly2, Ldb19-mediated recycling has not yet been described. Aly1, Aly2, and Ldb19 have overlapping functions as they each regulate the clathrin-independent and the clathrin-mediated endocytosis of Ste3, the yeast mating pheromone receptor in matα cells (Prosser et al., 2015). Additionally, as discussed in Chapter 1, there have been several instances in which other α-arrestins play a role in intracellular sorting. For example, Rod1 controls the intracellular trafficking of the lactate permease Jen1 (Becuwe and Leon, 2014). Additionally, the α-arrestins Bul1 and Bul2 promote both endocytosis and intracellular sorting (Golgi-to-endosome) of Gap1 (Helliwell et al., 2001; Merhi and Andre, 2012; Risinger and Kaiser, 2008; Soetens et al., 2001). A further detailed mechanism of how Aly1, Aly2, and Ldb19 traffic Kir2.1 is outlined in Chapter 3 and Appendix I.

Not only does this work identify dephosphorylation of Aly1 to be a negative regulator of Kir2.1 trafficking to the cell surface, but we also have shown that these select α-arrestins must bind to the yeast ubiquitin ligase Rsp5 in order to promote Kir2.1 localization to the plasma membrane. It has been well-defined that the \((^{(1/3)PXY}}\) motifs in α-arrestins bind the WW domains in Rsp5 (Alvarez, 2008; Gupta et al., 2007; Lin et al., 2008; Nikko and Pelham, 2009; Nikko et al., 2008; Rotin et al., 2000). Often times this interaction is associated with the ability of α-arrestins to partake in endocytosis as they act as a bridge between Rsp5 and membrane cargo proteins and
allow for Rsp5 to ubiquitinate these cargo membrane proteins thus stimulating endocytosis (Becuwe et al., 2012; Lin et al., 2008; Nikko and Pelham, 2009; Nikko et al., 2008). During this process, the α-arrestins themselves are also ubiquitinated (Becuwe et al., 2012; Lin et al., 2008; O'Donnell et al., 2015). α-Arrestin function is activated by mono-ubiquitination and so mutations in the lysines where this mono-ubiquitination occurs often impairs α-arrestin function (Alvaro et al., 2014; Becuwe et al., 2012; Lin et al., 2008; O'Donnell et al., 2015). Thus, it is hypothesized that monoubiquitination is critical in activation of α-arrestin-mediated protein trafficking (Ho et al., 2017). This is supported by work done by Becuwe et al. that shows mono-ubiquitination of Rod1 is essential for regulation of Jen1 trafficking (Becuwe et al., 2012) and Lin et al. which shows the need for monoubiquitination of Ldb19 to traffic Can1 (Lin et al., 2008). Lin et al. also showed that inability of Ldb19 to be monoubiquitinated inhibits its recruitment to both the Golgi and plasma membrane, suggesting monoubiquitination may regulate intracellular trafficking in addition to endocytosis (Lin et al., 2008). Our work identified monoubiquitination sites on Aly1 and Aly2, that when mutated and therefore unable to be monoubiquitinated, failed to rescue Kir2.1-dependent growth on low potassium and promote Kir2.1 localization to the plasma membrane. Using the PPXY-less mutants gives evidence that the Rsp5 interaction with the α-arrestins is essential for their ability to traffic Kir2.1 intracellularly.

Together, this work 1) identified a new cargo membrane protein, Kir2.1, for α-arrestins Aly1, Aly2, and Ldb10; 2) further elucidated the role of α-arrestins in intracellular sorting; 3) expanded knowledge of α-arrestin regulation via ubiquitination and phosphorylation status and; 4) employed the fluorogen activating protein to assess cell surface abundance of a membrane protein.
3.0 Defining the Intracellular Protein Trafficking Pathways Used by the α-Arrestins

3.1 Introduction

Controlling potassium levels in cells is essential for human health. Potassium channels play a key role in maintaining this homeostasis, including the inward rectifying potassium channels belonging to the KCNJ family. KCNJ member Kir2.1 is vital for maintaining optimal cardiac function as was discussed in detail in Chapter 1. Disruption of Kir2.1 function leads to diseases including short QT syndrome and Anderson-Tawil Syndrome (Perez-Riera et al., 2021; Plaster et al., 2001). Channel localization is a critical aspect of its function with defects in the trafficking of Kir2.1 to the cell surface associated with diseased states, however, there is still much to be learned about how the trafficking of this channel is controlled. One bottleneck for Kir2.1 trafficking studies of Kir2.1 is the inherent difficulties of working in primary cardiac cell and other native tissue systems. To overcome this obstacle, we use a yeast model system expressing this mammalian channel, which allows us to delineate trafficking factors important for Kir2.1 activity and localization. Using this system, we and others have defined conserved protein trafficking factors as regulators of Kir2.1 and another mammalian potassium channel Kir1.1 (ROMK), including ESCRT, retromer, and of most interest to us the α-arrestins (Hager et al., 2018; Kolb et al., 2014). The aim of this chapter is to define the role of α-arrestins in regulating Kir2.1 trafficking. Studies of α-arrestin function in yeast have demonstrated their role as protein trafficking adaptors that, in response to cell signaling events, link membrane cargo proteins to the ubiquitin ligase, Rsp5, and promote protein trafficking. In a targeted screen outlined in Chapter 2, we found that three specific α-arrestins—Aly1, Aly2, and Ldb19—promote Kir2.1 function at the plasma membrane. However,
the mechanism by which these α-arrestins operate, the factors needed for their regulation, and the conservation of this regulation in mammalian cells are as yet undefined. The following work aims to fill in these gaps.

We hypothesize that α-arrestins play a role in controlling the recycling of Kir2.1 from endosomal compartments, which increases Kir2.1 surface levels. To test this hypothesis, we deleted genes involved in intracellular sorting and endocytic trafficking pathways as well as regulators of the α-arrestins using our model system. We then assessed the ability of select α-arrestins to promote Kir2.1 surface localization in the absence of these key trafficking regulators. We have shown that the three α-arrestins—Aly1, Aly2, and Ldb19—each promote Kir2.1 function, however, when the machinery needed for α-arrestin regulated trafficking of Kir2.1 is deleted we anticipate loss of growth on low potassium medium and loss of these channels at the cell surface. Thus, this targeted screen should allow us to define the trafficking interval(s) required for α-arrestin-mediated trafficking of Kir2.1.

3.1.1 Identifying trafficking factors for α-arrestin-mediated trafficking of Kir2.1 in a yeast model system

In our yeast model system, Kir2.1 is ectopically expressed in yeast cells when the genes expressing the endogenous channels, TRK1 and TRK2, are deleted. We have previously shown that expression of Kir2.1 can rescue growth of these trk1Δ trk2Δ cells on low potassium medium and increase the intracellular K⁺ concentrations, demonstrating that it is a functional channel (see Chapter 2). As discussed previously, this model has facilitated the discovery of several regulators of Kir2.1 and its family member ROMK. For example, an AAA-ATPase (Cdc48) and an Hsp70
chaperone (Ssa1), both of which are required for ER-associated degradation (ERAD) have been shown to be required for maximal Kir2.1 degradation (Kolb et al., 2014). Furthermore, Kir2.1 is a substrate for ERAD, and this regulation is conserved from yeast to mammals (Kolb et al., 2014).

In parallel to this work, a genomic screen was conducted that took advantage of the yeast deletion collection and the ability of Kir2.1 to rescue growth on low potassium media. From this screen, a large number of candidates identified were involved in protein trafficking. This observation led to the work done in Chapter 2. In this chapter, we take a similar approach to Kolb et al., but here we focus on genes we hypothesize may be involved in the α-arrestin-mediated trafficking of Kir2.1.

Ultimately, this chapter aims to further define the pathway the α-arrestins use in their trafficking of Kir2.1. In order to do this, we have chosen to focus on two main facets of protein trafficking: the machinery for vesicle transport and signaling proteins that regulate α-arrestin function (Table 5 and Figure 13).

A role for the α-arrestins in intracellular trafficking has been previously reported. For example, α-arrestins Aly1 and Aly2 have been shown to regulate intracellular sorting of the general amino acid permease Gap1 in response to nutrient changes. Interestingly, this trafficking was dependent on the AP-1 complex and phosphorylation by the TORC1-dependent kinase, Npr1, for Aly2 (O'Donnell et al., 2010). Since both Aly1 and Aly2 have been shown to directly interact with the AP-1 complex, we decided to assess the α-arrestins ability to traffic Kir2.1 in the absence of AP-1 subunits (see below). Fellow clathrin adaptor complex, AP-2, is also of interest as this complex has been implicated to interact with β-arrestins and deemed important for β-arrestin-mediated endocytosis (Laporte et al., 2000). Another class of clathrin trafficking adaptors of interest are the GGAs. These are monomeric clathrin adaptor proteins, and have already been
associated with the α-arrestin-mediated trafficking of Jen1 and Dip5 (Becuwe and Leon, 2014; Nakayama and Wakatsuki, 2003).

As stated previously, when Kolb et al. screened for Kir2.1 regulators there were many trafficking hits. Based on this data, we chose to focus on the multivesicular body (MVB) sorting pathway via the ESCRT complex as we know this pathway is involved in Kir2.1 trafficking (Kolb et al., 2014). In short the MVB pathway begins at the endosome and transports cargo to the vacuole via the formation of multivesicular bodies. In order to examine the role that the MVB pathway has in Kir2.1 trafficking, we chose to delete ESCRT genes at varying steps in the MVB pathway since the ESCRT complex is responsible for MVB vesicle formation. For example, ESCRT-0 (Vps27) is involved in the initiation of MVBs, thus deletion of this gene will halt MVB formation at its start. We also interrupted the final state in MVB formation by deleting ESCRT-III (Vps4) (Babst, 2011). Another hit from the Kolb et al. screen, the retromer complex, is a significant player in vesicle pathways, specifically of retrograde transport from endosomes to the Golgi, and is important for Rod1 intracellular sorting (Becuwe and Leon, 2014; Burd and Cullen, 2014; Kolb et al., 2014).

In order to assess the relationship between endocytosis and the α-arrestin-mediated trafficking of Kir2.1, two key endocytic trafficking machinery was examined, Vrp1 and End3. Deletion of Vrp1 inhibits actin polymerization during endocytic internalization via disruption of the nucleation-promoting factor that initiates actin formation (Sun et al., 2006), while deletion of End3 inhibits coupling actin assembly in endocytic sites (Sun et al., 2017).

As discussed in Chapter 1, the phosphorylation status regulates α-arrestins. Thus, we elected to assess the effect two signaling proteins had on α-arrestin’s ability to traffic Kir2.1. The protein kinase, Npr1, has been shown phosphorylate Aly1 and Ldb19 (O’Donnell et al., 2010;
O'Donnell et al., 2013). Previously, this phosphorylation has been associated with the stabilization of membrane transporters by preventing the α-arrestins from mediating their ubiquitin-dependent degradation (De Craene et al., 2001; MacGurn et al., 2011; O'Donnell et al., 2010). The calcium/calmodulin-dependent protein phosphatase, calcineurin, has been shown to dephosphorylate Aly1 (O'Donnell et al., 2013). Moreover, calcineurin is present within the heart, making it a good candidate for α-arrestin-mediated Kir2.1 regulation (Strack et al., 1996).

Figure 13. Schematic representation of trafficking machinery assessed for Kir2.1-dependent trafficking

Table 5. List of gene deletions for targeted screen

<table>
<thead>
<tr>
<th>Complex</th>
<th>Gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-1</td>
<td>Apl2</td>
<td>Beta-adaptin of AP-1 that is involved in clathrin-dependent Golgi protein sorting (Kirchhausen, 2000a)</td>
</tr>
<tr>
<td>AP-1</td>
<td>Apl4</td>
<td>Gamma-adaptin of AP-1 that is involved in vesicle mediated transport (Kirchhausen, 2000a)</td>
</tr>
<tr>
<td>AP-2</td>
<td>Apl1</td>
<td>Beta-adaptin of AP-2 that is involved in vesicle mediated transport; endocytosis (Kirchhausen, 2000a)</td>
</tr>
<tr>
<td>AP-2</td>
<td>Apl3</td>
<td>Involved in vesicle mediated transport; endocytosis (Kirchhausen, 2000a)</td>
</tr>
<tr>
<td>Gga1</td>
<td>Gga1</td>
<td>Golgi-localized protein with homology to gamma-adaptin (Nakayama and Wakatsuki, 2003)</td>
</tr>
<tr>
<td>Complex</td>
<td>Gene</td>
<td>Function</td>
</tr>
<tr>
<td>-------------</td>
<td>---------</td>
<td>------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Gga2</td>
<td>Gga2</td>
<td>Golgi-localized protein with homology to gamma-adaptin (Nakayama and Wakatsuki, 2003)</td>
</tr>
<tr>
<td>ESCRT</td>
<td>Vps4</td>
<td>AAA-ATPase involved in multivesicular body (MVB) protein sorting (Babst, 2011)</td>
</tr>
<tr>
<td>ESCRT</td>
<td>Vps27</td>
<td>Endosomal protein required for recycling Golgi proteins, forming lumenal membranes and sorting ubiquitinated proteins destined for degradation (Babst, 2011)</td>
</tr>
<tr>
<td>Retromer</td>
<td>Vps35</td>
<td>Endosomal subunit of membrane-associated retromer complex that is required for retrograde transport (Burd and Cullen, 2014)</td>
</tr>
<tr>
<td>-</td>
<td>Vrp1</td>
<td>Verprolin, proline-rich actin-associated protein that promotes actin nucleation and endocytosis (Sun et al., 2006)</td>
</tr>
<tr>
<td>-</td>
<td>End3</td>
<td>EH domain-containing protein involved in endocytosis and actin cytoskeletal organization and cell wall morphogenesis (Sun et al., 2017)</td>
</tr>
<tr>
<td>Protein Kinase</td>
<td>Npr1</td>
<td>Protein kinase that stabilizes several plasma membrane amino acid transporters by antagonizing their ubiquitin-mediated degradation (Merhi and Andre, 2012)</td>
</tr>
<tr>
<td>Calcineurin</td>
<td>Cnb1</td>
<td>Calcineurin B; regulatory subunit of the phosphatase calcineurin (Rusnak and Mertz, 2000)</td>
</tr>
<tr>
<td>Sorting nexin</td>
<td>Snx4</td>
<td>Sorting nexin; involved in transport between post-Golgi endosomes to TGN (Ma and Burd, 2020)</td>
</tr>
<tr>
<td>F-box protein</td>
<td>Rey1</td>
<td>F-box protein used in recycling endocytosed proteins (Ma and Burd, 2020)</td>
</tr>
<tr>
<td>GTPase</td>
<td>Gtr2</td>
<td>Subunit of the GTPase, Gtr1-Gtr2, that activates TORC1 (Ma and Burd, 2020)</td>
</tr>
<tr>
<td>GTPase</td>
<td>Lst4</td>
<td>Subunit of GTPase, Lst4-Lst7, that activates Gtr2</td>
</tr>
</tbody>
</table>

### 3.2 Materials and Methods

#### 3.2.1 Yeast strains and growth conditions

Yeast strains used in this study and their construction are described in Table 6. Yeast were grown in synthetic complete (SC) medium prepared as described (Johnston et al., 1977). SC low-potassium medium was also prepared as described with the use of monosodium glutamate as a nitrogen source, the addition of 20 mM MES to maintain the pH, and the indicated amount of KCl (Kolb et al., 2014). Liquid medium was filter-sterilized, and for plated medium, 2% (w/v) agar was added prior to autoclaving. Plasmids were transformed into yeast via the lithium-acetate
method and selected for an appropriate SC medium. Yeast cells were grown at 30°C unless otherwise indicated. For growth assays on solid medium, 5-fold serial dilutions of saturated, overnight cultures (starting concentration of $1.0 \times 10^7$ cells/ml) were plated onto the indicated medium and grown for 2-6 days at 30°C.

### Table 6. List of strains used in Chapter 3

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>$trk1\Delta trk2\Delta$</td>
<td>MAT$\alpha$. $trk1\Delta::HIS3$ trk2$\Delta::HIS3$ $his3\Delta200$ leu2$\Delta1$ $ura3-52$ trp1-1 ade2</td>
<td>(Ko and Gaber, 1991; Nakamura and Gaber, 1998)</td>
</tr>
<tr>
<td>$trk1\Delta trk2\Delta$ $apl1\Delta$</td>
<td>MAT$\alpha$. $trk1\Delta::HIS3$ trk2$\Delta::HIS3$ $apl1\Delta::HPH$ $his3\Delta200$ leu2$\Delta1$ $ura3-52$ $trp1-1$ ade2</td>
<td>This study. This strain was derived from the $trk1\Delta trk2\Delta$ strain described inRefs. (Ko and Gaber, 1991; Nakamura and Gaber, 1998). The $apl1$ coding region was replaced by the hygromycin resistance cassette as described in (Ko and Gaber, 1991).</td>
</tr>
<tr>
<td>$trk1\Delta trk2\Delta$ $apl3\Delta$</td>
<td>MAT$\alpha$. $trk1\Delta::HIS3$ trk2$\Delta::HIS3$ $apl3\Delta::HPH$ $his3\Delta200$ leu2$\Delta1$ $ura3-52$ $trp1-1$ ade2</td>
<td>This study. This strain was derived from the $trk1\Delta trk2\Delta$ strain described inRefs. (Ko and Gaber, 1991; Nakamura and Gaber, 1998). The $apl3$ coding region was replaced by the hygromycin resistance cassette as described in (Ko and Gaber, 1991).</td>
</tr>
<tr>
<td>$trk1\Delta trk2\Delta$ $apl2\Delta$</td>
<td>MAT$\alpha$. $trk1\Delta::HIS3$ trk2$\Delta::HIS3$ $apl2\Delta::HPH$ $his3\Delta200$ leu2$\Delta1$ $ura3-52$ $trp1-1$ ade2</td>
<td>This study. This strain was derived from the $trk1\Delta trk2\Delta$ strain described inRefs. (Ko and Gaber, 1991; Nakamura and Gaber, 1998). The $apl2$ coding region was replaced by the hygromycin resistance cassette as described in (Ko and Gaber, 1991).</td>
</tr>
<tr>
<td>Strain</td>
<td>Genotype</td>
<td>Source</td>
</tr>
<tr>
<td>-------------</td>
<td>---------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>trk1Δ trk2Δ</td>
<td>MATα trk1Δ:: HIS3 trk2Δ::HIS3 gga1Δ::HPH his3Δ200 leu2Δ1 ura3-52 trp1-1 ade2</td>
<td>This study. This strain was derived from the trk1Δ trk2Δ strain described in Refs. (Ko and Gaber, 1991; Nakamura and Gaber, 1998). The gga1 coding region was replaced by the hygromycin resistance cassette as described in (Ko and Gaber, 1991).</td>
</tr>
<tr>
<td>gga2Δ</td>
<td>MATα trk1Δ:: HIS3 trk2Δ::HIS3 gga2Δ::HPH his3Δ200 leu2Δ1 ura3-52 trp1-1 ade2</td>
<td>This study. This strain was derived from the trk1Δ trk2Δ strain described in Refs. (Ko and Gaber, 1991; Nakamura and Gaber, 1998). The gga2 coding region was replaced by the hygromycin resistance cassette as described in (Ko and Gaber, 1991).</td>
</tr>
<tr>
<td>trk1Δ trk2Δ</td>
<td>MATα trk1Δ:: HIS3 trk2Δ::HIS3 vps4Δ::HPH his3Δ200 leu2Δ1 ura3-52 trp1-1 ade2</td>
<td>This study. This strain was derived from the trk1Δ trk2Δ strain described in Refs. (Ko and Gaber, 1991; Nakamura and Gaber, 1998). The vps4 coding region was replaced by the hygromycin resistance cassette as described in (Ko and Gaber, 1991).</td>
</tr>
<tr>
<td>vps4Δ</td>
<td>MATα trk1Δ:: HIS3 trk2Δ::HIS3 vps4Δ::HPH his3Δ200 leu2Δ1 ura3-52 trp1-1 ade2</td>
<td>This study. This strain was derived from the trk1Δ trk2Δ strain described in Refs. (Ko and Gaber, 1991; Nakamura and Gaber, 1998). The vps4 coding region was replaced by the hygromycin resistance cassette as described in (Ko and Gaber, 1991).</td>
</tr>
<tr>
<td>vps27Δ</td>
<td>MATα trk1Δ:: HIS3 trk2Δ::HIS3 vps27Δ::HPH his3Δ200 leu2Δ1 ura3-52 trp1-1 ade2</td>
<td>This study. This strain was derived from the trk1Δ trk2Δ strain described in Refs. (Ko and Gaber, 1991; Nakamura and Gaber, 1998). The vps27 coding region was replaced by the hygromycin resistance cassette as described in (Ko and Gaber, 1991).</td>
</tr>
<tr>
<td>trk1Δ trk2Δ</td>
<td>MATα trk1Δ:: HIS3 trk2Δ::HIS3 vps35Δ::HPH his3Δ200 leu2Δ1 ura3-52 trp1-1 ade2</td>
<td>This study. This strain was derived from the trk1Δ trk2Δ strain described in Refs. (Ko and Gaber, 1991; Nakamura and Gaber, 1998). The vps35 coding region was replaced by the hygromycin resistance cassette as described in (Ko and Gaber, 1991).</td>
</tr>
<tr>
<td>Strain</td>
<td>Genotype</td>
<td>Source</td>
</tr>
<tr>
<td>---------------</td>
<td>--------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td>$trk_{1\Delta \ trk_{2\Delta}}$ $vrp_{1\Delta}$</td>
<td>MAT$\alpha$ $trk_{1\Delta}$::HIS3 $trk_{2\Delta}$::HIS3 $vrp_{1\Delta}$::HPH $his_{3\Delta}200$ $leu_{2\Delta}1$ $ura_{3\Delta}52$ $trp_{1\Delta}$ $ade_{2}$</td>
<td>This study. This strain was derived from the $trk_{1\Delta \ trk_{2\Delta}}$ strain described in Refs. (Ko and Gaber, 1991; Nakamura and Gaber, 1998). The $vrp_{1\Delta}$ coding region was replaced by the hygromycin resistance cassette as described in (Ko and Gaber, 1991).</td>
</tr>
<tr>
<td>$trk_{1\Delta \ trk_{2\Delta}}$ $end_{3\Delta}$</td>
<td>MAT$\alpha$ $trk_{1\Delta}$::HIS3 $trk_{2\Delta}$::HIS3 $end_{3\Delta}$::HPH $his_{3\Delta}200$ $leu_{2\Delta}1$ $ura_{3\Delta}52$ $trp_{1\Delta}$ $ade_{2}$</td>
<td>This study. This strain was derived from the $trk_{1\Delta \ trk_{2\Delta}}$ strain described in Refs. (Ko and Gaber, 1991; Nakamura and Gaber, 1998). The $end_{3\Delta}$ coding region was replaced by the hygromycin resistance cassette as described in (Ko and Gaber, 1991).</td>
</tr>
<tr>
<td>$trk_{1\Delta \ trk_{2\Delta}}$ $npr_{1\Delta}$</td>
<td>MAT$\alpha$ $trk_{1\Delta}$::HIS3 $trk_{2\Delta}$::HIS3 $npr_{1\Delta}$::HPH $his_{3\Delta}200$ $leu_{2\Delta}1$ $ura_{3\Delta}52$ $trp_{1\Delta}$ $ade_{2}$</td>
<td>This study. This strain was derived from the $trk_{1\Delta \ trk_{2\Delta}}$ strain described in Refs. (Ko and Gaber, 1991; Nakamura and Gaber, 1998). The $npr_{1\Delta}$ coding region was replaced by the hygromycin resistance cassette as described in (Ko and Gaber, 1991).</td>
</tr>
<tr>
<td>$trk_{1\Delta \ trk_{2\Delta}}$ $cnb_{1\Delta}$</td>
<td>MAT$\alpha$ $trk_{1\Delta}$::HIS3 $trk_{2\Delta}$::HIS3 $cnb_{1\Delta}$::HPH $his_{3\Delta}200$ $leu_{2\Delta}1$ $ura_{3\Delta}52$ $trp_{1\Delta}$ $ade_{2}$</td>
<td>This study. This strain was derived from the $trk_{1\Delta \ trk_{2\Delta}}$ strain described in Refs. (Ko and Gaber, 1991; Nakamura and Gaber, 1998). The $cnb_{1\Delta}$ coding region was replaced by the hygromycin resistance cassette as described in (Ko and Gaber, 1991).</td>
</tr>
<tr>
<td>$trk_{1\Delta \ trk_{2\Delta}}$ $snx_{4\Delta}$</td>
<td>MAT$\alpha$ $trk_{1\Delta}$::HIS3 $trk_{2\Delta}$::HIS3 $snx_{4\Delta}$::HPH $his_{3\Delta}200$ $leu_{2\Delta}1$ $ura_{3\Delta}52$ $trp_{1\Delta}$ $ade_{2}$</td>
<td>This study. This strain was derived from the $trk_{1\Delta \ trk_{2\Delta}}$ strain described in Refs. (Ko and Gaber, 1991; Nakamura and Gaber, 1998). The $snx_{4\Delta}$ coding region was replaced by the hygromycin resistance cassette as described in (Ko and Gaber, 1991).</td>
</tr>
<tr>
<td>Strain</td>
<td>Genotype</td>
<td>Source</td>
</tr>
<tr>
<td>-----------------</td>
<td>--------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>trk1Δ trk2Δ</td>
<td>MATα trk1Δ::HIS3 trk2Δ::HIS3 rcy1Δ::HPH his3Δ200 leu2Δ1 ura3-52 trp1-1 ade2</td>
<td>This study. This strain was derived from the trk1Δ trk2Δ strain described in Refs. (Ko and Gaber, 1991; Nakamura and Gaber, 1998). The rcy1 coding region was replaced by the hygromycin resistance cassette as described in (Ko and Gaber, 1991).</td>
</tr>
<tr>
<td>trk1Δ trk2Δ</td>
<td>MATα trk1Δ::HIS3 trk2Δ::HIS3 gtr2Δ::HPH his3Δ200 leu2Δ1 ura3-52 trp1-1 ade2</td>
<td>This study. This strain was derived from the trk1Δ trk2Δ strain described in Refs. (Ko and Gaber, 1991; Nakamura and Gaber, 1998). The gtr2 coding region was replaced by the hygromycin resistance cassette as described in (Ko and Gaber, 1991).</td>
</tr>
<tr>
<td>trk1Δ trk2Δ</td>
<td>MATα trk1Δ::HIS3 trk2Δ::HIS3 lst4Δ::HPH his3Δ200 leu2Δ1 ura3-52 trp1-1 ade2</td>
<td>This study. This strain was derived from the trk1Δ trk2Δ strain described in Refs. (Ko and Gaber, 1991; Nakamura and Gaber, 1998). The lst4 coding region was replaced by the hygromycin resistance cassette as described in (Ko and Gaber, 1991).</td>
</tr>
</tbody>
</table>

### 3.2.2 Plasmids and DNA manipulations

Plasmids used in this study and details of their construction are described in Table 7. PCR amplifications were performed using Phusion High Fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, MA), and all constructs generated were verified using Sanger sequencing (Genewiz, South Plainfield, NJ).
Table 7. Plasmids used in Chapter 3

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Description/References</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRS415-TEF1pr</td>
<td>TEF1pr CEN LEU2</td>
<td>(Brette and Orchard, 2003)</td>
</tr>
<tr>
<td>pRS415-TEF1pr-Kir2.1-HA</td>
<td>TEF1pr-Kir2.1-HA CEN LEU2</td>
<td>(Curran et al., 2014)</td>
</tr>
<tr>
<td>pRS426</td>
<td>2 μm URA3</td>
<td>(de Boer et al., 2010)</td>
</tr>
<tr>
<td>pRS426-ALY1</td>
<td>ALY1pr-ALY1 2 μm URA3</td>
<td>(Hibino et al., 2010)</td>
</tr>
<tr>
<td>pRS426-ALY2</td>
<td>ALY2pr-ALY2 2 μm URA3</td>
<td>(Hibino et al., 2010)</td>
</tr>
<tr>
<td>pRS426-LDB19</td>
<td>LDB19pr-LDB19 2 μm URA3</td>
<td>(Clarke et al., 2010)</td>
</tr>
<tr>
<td>pRS426-ART5</td>
<td>ART5pr-ART5 2 μm URA3</td>
<td>(Nichols and Lopatin, 1997)</td>
</tr>
</tbody>
</table>

3.3 Results and Discussion

3.3.1 Kir2.1 function is regulated by the AP-1 complex

The AP-1 complex is a clathrin adaptor complex that traffics between the Golgi and endosomes (Bonifacino and Traub, 2003; Ma et al., 2011). Deletion of the beta (apl2Δ) and gamma (apl4Δ) subunits in the AP-1 complex allowed us to assess its relationship with Kir2.1. Deletion of each of these subunits increased growth on low potassium medium (5 mm KCl) in a Kir2.1-dependent manner (Figure 13A, row 5 and 9). Furthermore, the ability of Aly1 to increase growth on low potassium medium (5 mM KCl) in the trk1Δ trk2Δ apl2Δ strain was slightly decreased compared to the trk1Δ trk2Δ background (Figure 13A, row 2 and 6) whereas Aly2 and Ldb19-mediated trafficking of Kir2.1 appeared to be unaffected. This suggests the ability of Aly1 to traffic Kir2.1 is at least partially dependent on the AP-1 complex.

As discussed previously, Kir2.1 is known to directly interact with AP-1 (Bonifacino and Traub, 2003; Ma et al., 2011) and the Kir2.1-Δ314-315 mutant that prevents this interaction results in retention of Kir2.1 in the Golgi, causing severe ATS phenotype (Ma et al., 2011). Additionally,
Aly1 and Aly2 both interact with the AP-1 complex (O'Donnell et al., 2010). Interestingly, Aly2-mediated trafficking of Gap1 is dependent upon AP-1 (O'Donnell et al., 2010). Together, these data support a model where Kir2.1 trafficking is dependent on AP-1, and both Aly1 and Aly2 may use the AP-1 to traffic distinct membrane cargo.

Figure 14. AP-1 Complex regulates Kir2.1 trafficking

(A) Growth of serial dilutions of trk1Δ trk2Δ cells containing the indicated plasmids on SC medium lacking leucine and uracil and containing the indicated added amounts of KCl is shown after 2 days.

3.3.2 Kir2.1 function and the AP-2 complex

To analyze the role the AP-2 complex plays in Kir2.1 trafficking we deleted the beta (apl1Δ) and gamma (apl3Δ) subunits and assessed Kir2.1 function. In contrast to our AP-1 results, deletion of AP-2 subunits did not rescue Kir2.1-dependent growth on low potassium medium (Figure 12A, row 5 and 9). However, we did see a slight decrease in the ability of Aly2 to rescue growth on low potassium (5 mM KCl) medium (Figure 12A, row 3, 7, and 11) suggesting that Aly2-mediated trafficking of Kir2.1 may benefit from functional AP-2 complex.

As with the AP-1 complex, the AP-2 complex plays a role in the trafficking of the Kir family. For example, family member Kir2.3 is internalized from the PM in an AP-2-dependent manner in HEK293T cells (Ortega et al., 2012). Specifically, the knockdown of the AP-2 complex
diminished Kir3.2 endocytosis (Mason et al., 2008). Furthermore, several ties between β-arrestins trafficking and the AP-2 complex have been defined (Shenoy and Lefkowitz, 2003), raising the possibility that α-arrestins may also be able to interact AP-2.

**Figure 15. AP-2 complex does not affect Kir2.1 trafficking**

(A) Growth of serial dilutions of trk1Δ trk2Δ cells containing the indicated plasmids on SC medium lacking leucine and uracil and containing the indicated added amounts of KCl is shown after 2 days.

### 3.3.3 Kir2.1 function is regulated by ESCRT

Consistent with previous work, deletion of ESCRT components increased Kir2.1-dependent growth on low potassium medium (Figure 15A, row 5 and 9). However, this increased growth made it difficult to assess the dependence of the ESCRT complex on the α-arrestin-mediated trafficking of Kir2.1 (Figure 15A). Interestingly, the ability of Aly2 to improve growth on low potassium is improved in the absence of ESCRTs (Figure 15A, row 7) compared to vector alone. This finding, coupled with the AP-2 result above, suggest that Aly2 trafficking of Kir2.1 may depend on a separate pool of Kir2.1, perhaps one that is localized to endosomes, that is not accessed by the other α-arrestins. To explain this idea further, we find that Aly2-mediated growth on low potassium is impaired in the absence of the AP-2 complex, which would impede Kir2.1 trafficking to the endosomes from the PM. In the ESCRT mutations, specifically vps4Δ cells, there
is likely retention of Kir2.1 in endosomal pools as cargo backs up into class E compartments and early endosomes when ESCRTs are impeded. In this scenario, we see increased ability of Aly2 to rescue growth on low potassium medium, which is consistent with the idea that Aly2 is able to stimulate recycling from an endosome pool.

![Figure 16. Kir2.1 is regulated by ESCRT](image)

(A) Growth of serial dilutions of *trk1Δ trk2Δ* cells containing the indicated plasmids on SC medium lacking leucine and uracil and containing the indicated added amounts of KCl is shown after 2 days.

### 3.3.4 Retromer is a regulator of Kir2.1

When Retromer subunit, Vps35, was deleted there was increased Kir2.1-dependent growth on low potassium medium (Figure 16A, row 5), which is consistent with what was observed previously (Kolb et al., 2014). However, interestingly, loss of Vps35 reduces Aly1-mediated growth on low potassium (0, 5, and 10 mM KCl) (Figure 16A, row 6). This suggests that Aly1’s ability to traffic Kir2.1 is dependent on the retromer complex, suggesting that Aly1 accesses a pool of Kir2.1 that is either in the Golgi or in an endosomal recycling pathway dependent upon Retromer.
3.3.5 Kir2.1 function is regulated by Vrp1 and End3

To disrupt clathrin-mediated endocytosis in a more general way, we deleted either End3, results in the failure to assemble actin at endocytic sites and impaired endocytosis (Sun et al., 2015) or Vrp1, which promotes actin nucleation and similarly disrupts endocytosis (Sun et al., 2006). Inhibition of endocytosis via End3 gave robust Kir2.1-dependent growth on low potassium, and this made it difficult to assess any dependence on the α-arrestins on this mechanism (Figure 16, row 5-8). Alternatively, when Vrp1 was deleted we did not see any increase of Kir2.1-dependent growth compared to WT cells with a vector control. This suggests that the End3 and Vrp1 mutants are not equivalent in their capacity to alter Kir2.1 trafficking. When examining α-arrestin regulation, we found that Aly2 was unable to restore growth on low potassium medium (5mM KCl) in the vrp1Δ cells (Figure 17, row 11). These results are consistent with the idea, described above, that Aly2-mediated trafficking of Kir2.1 operates on a post-endocytic pool of Kir2.1.
Inhibition of endocytosis affects Kir2.1 trafficking

Figure 18. Inhibition of endocytosis affects Kir2.1 trafficking

(A) Growth of serial dilutions of trk1Δ trk2Δ cells containing the indicated plasmids on SC medium lacking leucine and uracil and containing the indicated added amounts of KCl is shown after 2 days.

3.3.6 Aly2-mediated trafficking of Kir2.1 is dependent on Npr1

Previously, O’Donnell et al. showed that Aly2-dependent trafficking of Gap1 required Npr1, a protein kinase known to phosphorylate Aly2 and other α-arrestins (O’Donnell et al., 2010). Moreover, this work and that of others support the notion that phosphorylation acts as a switch to control the α-arrestins’ function in intracellular sorting or in endocytosis. Consistent with this notion, we saw that when NPR1 was deleted and thus preventing its phosphorylation of Aly2, Aly2 was no longer able to rescue growth on low potassium (5 mM KCl) in a Kir2.1-dependent manner (Figure 18, row 7).
Figure 19. Deletion of Npr1 inhibits Aly2-mediated trafficking of Kir2.1

Growth of serial dilutions of trk1Δ trk2Δ cells containing the indicated plasmids on SC medium lacking uracil and leucine and containing the indicated added concentrations of KCl is shown after 3 days of incubation.

3.3.7 GGA regulation of Kir2.1

The GGA proteins are a family of clathrin adaptors that have previously been connected to the α-arrestin-mediated trafficking of Jen1 and Dip5, the lactate and aspartic acid transporters respectively (Becuwe and Leon, 2014; Nakayama and Wakatsuki, 2003). Specifically, in the absence of GGAs, Jen1 is retained in the Golgi. (Becuwe and Leon, 2014) In cells lacking Gga2, there was a robust increase in growth on low potassium medium. Unfortunately, this made it impossible to assess the role of α-arrestins in this trafficking pathway. These data suggest that Gga2 is a regulator of Kir2.1, and that Kir2.1 trafficking to or retention at the PM is more robust in the absence of Gga2 (Figure 19A, row 9-12). Interestingly, and in marked contrast to Gga1, loss of just Gga1 had no impact on Kir2.1-dependent growth nor did it influence the α-arrestin function.
Figure 20. Kir2.1 is regulated by Gga1

Growth of serial dilution assays of trk1Δ trk2Δ cells, trk1Δ trk2Δ gga1Δ cells, or trk1Δ trk2Δ gga2Δ cells containing the indicated plasmids on SC medium lacking leucine and uracil and containing the indicated added amount of KCl is shown after 2 days.

Together, these data help us to begin to understand the trafficking pathways α-arrestins use to usher Kir2.1 to the cell surface. Currently, studies utilizing the FAP technology are being done to measure the PM localization of Kir2.1 in these backgrounds. This work will allow us to define specific machinery responsible for Kir2.1 regulation and how this machinery impacts α-arrestin-mediated trafficking of Kir2.1.

Table 8. Summary of genetic ineractions between trafficking machinery and the α-arrestins in controlling Kir2.1-dependent growth on low potassium

<table>
<thead>
<tr>
<th>Machinery</th>
<th>Kir2.1</th>
<th>Aly1</th>
<th>Aly2</th>
<th>Ldb19</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-1 Complex</td>
<td>Inhibits</td>
<td>Dependent</td>
<td>Inhibits</td>
<td>Inhibits</td>
</tr>
<tr>
<td>AP-2 Complex</td>
<td>Independent</td>
<td>Independent</td>
<td>Dependent</td>
<td>Independent</td>
</tr>
<tr>
<td>ESRCT</td>
<td>Inhibits</td>
<td>Independent</td>
<td>Inhibits</td>
<td>Inhibits</td>
</tr>
<tr>
<td>Retromer</td>
<td>Inhibits</td>
<td>Dependent</td>
<td>Inhibits</td>
<td>Inhibits</td>
</tr>
<tr>
<td>Vrp1</td>
<td>Independent</td>
<td>Independent</td>
<td>Dependent</td>
<td>Independent</td>
</tr>
<tr>
<td>End3</td>
<td>Inhibits</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Npr1</td>
<td>Independent</td>
<td>Independent</td>
<td>Dependent</td>
<td>Independent</td>
</tr>
<tr>
<td>Gga1</td>
<td>Independent</td>
<td>Independent</td>
<td>Independent</td>
<td>Independent</td>
</tr>
<tr>
<td>Gga2</td>
<td>Inhibits</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
</tr>
</tbody>
</table>

*Inhibits= better growth; dependent= worse growth; independent= same growth; n/d= not defined.
4.0 Fluorogen Activating Proteins as a Tool for Quantitative Protein Trafficking Studies In Yeast

The contents of this chapter are adapted from a manuscript that is in preparation for submission to Molecular Biology of the Cell.

4.1 Introduction

To ensure survival, cells must precisely regulate protein distribution within membrane-bound organelles. Specifically, membrane proteins are incorporated into transport vesicles and trafficked between organelles. Additionally, at the PM, membrane proteins are continuously trafficked to and from the cell surface through selective protein trafficking in response to environmental cues. Inability to maintain this regulatory mechanism results in disease (Aridor and Hannan, 2000; Howell et al., 2006; Hung and Link, 2011; Yarwood et al., 2020). For example, in rare instances, diabetes mellitus can be caused by the inability of insulin receptors to traffic to the PM (Kadowaki et al., 1991). Additionally, as discussed previously in Chapter 1, improper trafficking of Kir2.1 results in arrhythmias and increased risk for heart failure (Ma et al., 2011).

To better understand the underlying origins of diseases involving membrane proteins, it is critical to distinguish between changes in protein activity and protein localization (Aridor and Hannan, 2000; Perkins and Bruchez, 2020; Yarwood et al., 2020). Using the cystic fibrosis transmembrane conductance regulator (CFTR) as an example, it is important to distinguish between mutations that affect its synthesis, folding, gating, conductance, or trafficking as each
aberration requires different interventions to improve function (Ahner et al., 2007; Cheng et al., 1990; Coppinger et al., 2012; Farinha and Amaral, 2005; Jensen et al., 1995; Koulov et al., 2010; Lopes-Pacheco, 2016, 2019; Meacham et al., 1999; Moskowitz et al., 2008; Wang et al., 2006). Therefore, the ability to execute quantitative studies of endocytosis and intracellular protein trafficking is an invaluable tool that can be used to define the mechanism underlying these disease states.

Many techniques have been developed to provide quantitative readouts of protein trafficking, with an emphasis on endocytic assays. For example, in many instances cell-surface biotinylation assays and ligand-labelling have been used to shed light on the endocytosis and recycling of select proteins of interest (Bohme and Beck-Sicking, 2009; Chen et al., 2011; Nishimura and Sasaki, 2008; Tham and Moukhles, 2017). Though these techniques offer significant insight into protein trafficking, they do come with limitations; For example, cell-surface biotinylation can aberrantly trigger endocytosis as a result of receptor crosslinking (Dundas et al., 2013) and can miscalculate the abundance of membrane proteins due to decreased binding to streptavidin (Tham and Moukhles, 2017). In addition, cell-surface biotinylation can’t be used in all cell types. Specifically, in several yeast species, streptavidin will non-specifically bind to the cell wall (Masuoka et al., 2002). This non-specific binding can also be seen in human B cells and myeloid cells, where streptavidin non-specifically binds to some surface proteins (Cole et al., 1987). Extraction of membrane proteins in certain cell types also present an obstacle to surface biotinylation. In yeast, their thick cell walls which increase in size during stress conditions and in late stationary growth phase make this extraction inherently difficult (Klis et al., 2002; Mukherjee et al., 2020; Powell et al., 2003; Smith et al., 2000; Walton, 1979).
To avoid these limitations, ligand labels and derivatized ligands can be used. Modified ligands used in these approaches must be impermeant to the cell surface, engage the receptor equivalently to the native ligand, remain bound to monitor localization, and maintain photo stability (Jonker et al., 2020; Komatsu et al., 2011; Leng et al., 2017; Los et al., 2008). This was successfully done in yeast when radioactive α-factor was used to stimulate the trafficking of the G-protein-coupled receptor (GPCR) Ste2 (Dunn and Hicke, 2001; Hicke et al., 1998; Shih et al., 2002; Toshima et al., 2009; Zanolari and Riezman, 1991). More recently, fluorescently-tagged α-factor was also used to monitor internalization of Ste2 (Toshima et al., 2006). α-Factor is the mating pheromone that binds Ste2 and activates the signal transduction pathway that induces yeast cells mating. Both radioactive and fluorescently tagged α-factor have been used to assess the dynamics of Ste2 after ligand binding and consequent internalization (Toshima et al., 2006). Similar methods in mammalian cells have been used to identify how sphingolipids traffic and interact with lipids and proteins (Schwarzmann et al., 2014). Together these tools have provided insight into these trafficking pathways, but they are not easily transferable to other membrane cargos and some modified ligands show reduced binding affinities that influence their ability to stimulate trafficking and/or signaling (Ding et al., 2002; Sridharan et al., 2014).

Since the discovery of green fluorescent proteins (GFP) in the 1960s, FPs and their derivatives have been widely used to label and monitor protein localization. FPs have led to revolutionary changes in our understanding of cell biology and protein trafficking, but they come with limitations. FPs can make it difficult to selectively label a specific cellular compartment due to background fluorescence resulting from the fluorescence intensity. For example, GFP is difficult to degrade, resulting in a bright vacuolar fluorescence which can mask other pools of GFP-tagged proteins. Derivatives of FPs, like the pH-sensitive FP pHluorin, can be used for quantitative...
endocytic assays since its fluorescence is quenched in intracellular organelles like MVBs and upon delivery to the lysosome/vacuole (Prosser et al., 2016). Although this FP derivative can be very helpful in alleviating intracellular accumulation of FP fluorescence, since in acidic environments the fluorescence is quenched, it is limited since the quenching is reliant on the pH of the organelles it traffics to. Additionally, because of the quenched fluorescence, pHfluorin-tagged proteins cannot be used to study recycling.

Advancements in the field led to the first generation of biconjugate tags, which form covalent bonds with target chemical fluorophores. The first of its kind, F1AsH/ReAsh probes used a tetracysteine peptide tag to visualize mutant calmodulin in HeLa cells (Griffin et al., 1998). When the fused peptide tag binds the probe, light is emitted; however, these probes have a high affinity for cysteine-rich proteins which can result in significant background fluorescence (Gallo, 2020; Griffin et al., 1998; Zurn et al., 2010). Popular tags like the Halo-, CLIP-, SNAP-, LAP- (localization and affinity purification) and BL-tags (β-lactamase tag) engineer enzymes to be genetic tags. These tags form irreversible bonds with fluorescently labeled ligands (Gautier et al., 2008; Keppler et al., 2003; Los et al., 2008; Watanabe et al., 2010; Yao et al., 2012). These tags are fused to a protein of interest and bind fluorescent dyes (Casler and Glick, 2020; Chen et al., 2005; Fernandez-Suarez et al., 2007; Gallo, 2020; Gautier et al., 2008; Griffin et al., 1998; Keppler et al., 2003; Keppler et al., 2004; Luedtke et al., 2007).

In recent years, fluorescent imaging has continued to evolve resulting in a second generation of biconjugate tags. Best known and the focus of this chapter, are the fluorogen activating proteins (FAPs). FAPs further advance studies of protein trafficking as they provide the ability to selectively label specific membrane pools, enhance spatial-temporal visualization, and provide flexible imaging parameters (Boeck and Spencer, 2017; Emmerstorfer-Augustin et al.,
FAP tags consist of a single chain antibody (SCA) that is fused to the protein of interest. The variable region of the SCA displays a high affinity for non-fluorescent dyes called fluorogens. When unbound, neither the SCA nor the fluorogen is fluorescent (Figure 20) (Fisher et al., 2010; Gallo, 2020; Perkins and Bruchez, 2020; Szent-Gyorgyi et al., 2008). However, when bound, the FAP-fluorogen complex, which forms via non-covalent interactions, restricts the fluorogen conformation and results in light emission upon excitation at a specific wavelength (Gallo, 2020; Lee et al., 1986; Shank et al., 2013; Silva et al., 2007). FAP technology yields a similar sensitivity and fluorescence intensity as conventional fluorescent probes such as eGFP and mCherry (Szent-Gyorgyi et al., 2013), but offers a better signal-to-noise ratio since unbound fluorogens are quenched, making it unnecessary to wash away unbound fluorogen in some applications (Perkins and Bruchez, 2020). In fact, there is potential that this interaction can be removed upon washing (Perkins and Bruchez, 2020). Other advantages of the FAP include: 1) Applications accommodate multi-fluorescent tags in these experiments, since fluorogens can be derived from a variety of dyes, including malachite green (MG) dye, which is the focus of this chapter, 2) The dye can be derivatized for a range of uses, important to this chapter is the use of either cell-permeant or -impermeant fluorogens (Figure 20, B and C), and 3) There are a range of available FAP dye derivatives including FRET and pH sensitive dyes (Perkins and Bruchez, 2020) as well as cell-impermeant fluorogens that are able to distinguish membrane proteins at the cell surface from intracellular proteins. This allows for selective assessment of endocytosis and post-endocytic trafficking. This approach can also be used to measure the return rate of a protein to the cell surface (Szent-Gyorgyi et al., 2013). Notably, FAPs have been used to examine the endocytosis, sorting, and recycling of GABA<sub>A</sub> receptors in neurons (Lorenz-Guertin et al., 2017), the endocytosis of β2-
adrenergic receptors (β2AR) in NIH3T3 fibroblasts (Holleran et al., 2010), β2AR shuffling to and from the PM in U937 lymphoma cells (Fisher et al., 2010) and mutant CFTR trafficking to the cell surface in human bronchial epithelial cells and HEK293T cells (Holleran et al., 2012). If using different derivatives, permeant fluorogens can be used in combination with impermeant fluorogens to identify the total cellular pool of tagged protein versus the cell surface pool (Naganbabu et al., 2016; Perkins and Bruchez, 2020; Pratt et al., 2015; Yan et al., 2015).

Although widely used, the FAP technology is not commonly adapted for use in the budding yeast Saccharomyces cerevisiae, which is surprising since the SCAs were actually developed using yeast screens (Feldhaus et al., 2003; Szent-Gyorgyi et al., 2008). Instead, FAPs are usually used in mammalian systems. Only recently have we and others employed the FAP technology to successfully track the sorting of membrane proteins in yeast (Emmerstorfer-Augustin et al., 2018; Hager et al., 2018). We were the first to publish that FAP can be effectively applied in yeast to quantitatively define the abundance of a plasma membrane protein. In this study, we ectopically expressed the FAP-tagged cardiac potassium channel Kir2.1 in Saccharomyces cerevisiae. This technology helped us in identifying the α-arrestins as novel molecular players regulating PM abundance of Kir2.1, which is the focus of Chapter 2 (Hager et al., 2018). A subsequent study used the FAP technology to quantitatively assess the localization of the G-protein coupled receptor Ste2. This revealed that de novo synthesis of Ste2, rather than site-specific endocytosis, resulted in Ste2 accumulation at the shmoo tip in MATa cells treated with α-factor (Emmerstorfer-Augustin et al., 2018) and confirmed the importance of α-arrestins in regulating Ste2 dynamics, consistent with previously reported work (Alvaro et al., 2014; Ayscough and Drubin, 1998; Moore et al., 2008).

These studies support the potential use of FAPs to visualize dynamic and complex sorting mechanisms in yeast and provide new spatial-temporal resolution to trafficking activities. With
these possibilities in mind, the following work aimed to enhance the performance and accessibility of the FAP technology in yeast. To achieve this objective, we: 1) codon optimized the SCA FAPα2 sequence for expression in *Saccharomyces cerevisiae*; 2) created a collection of FAP-tagged cloning vectors using optimized FAP, which we have made available to the scientific community to aid in protein trafficking research; 3) generated a suite of FAP-tagged cellular markers for use in yeast co-localization studies.

4.2 Materials and Methods

4.2.1 Yeast strains and growth conditions

Yeast were grown in synthetic complete (SC) medium prepared as described (Johnston et al., 1977) and supplemented with 5M sodium hydroxide until a pH of 6.6 was reached unless otherwise indicated (See pH optimization methods). Liquid medium was filter sterilized and for plate medium, 2% w/v agar was added prior to autoclaving. The lithium-acetate method was used for plasmid transformations (Ausubel, 1991) and selection of plasmids was assured using the appropriate SC drop-out medium. Yeast cells were grown at 30°C unless otherwise indicated.

4.2.2 Plasmids and DNA manipulations

Plasmids used in this study and details of their construction or origin are described in Table 9. PCR amplifications were performed using Phusion High Fidelity DNA polymerase (ThermoFisher Scientific, Waltham, MA). All constructs generated were verified using Sanger
sequencing (Genewiz, South Plainfield, NJ). Plasmid maps were generated using SnapGene software (Insightful Science, Chicago, IL). Geneblocs of the original and optimized sequence of FAP were obtained from GeneWiz (Genewiz, South Plainfield, NJ). Codon optimization was done using the JAVA Codon Adaptation Tool (JCat) (Grote et al., 2005).

Table 9. Plasmids used in Chapter 4

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Description/References</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRS415-TEF1pr</td>
<td>TEF1pr CEN LEU2</td>
<td>(Mumberg et al., 1995)</td>
</tr>
<tr>
<td>pRS415-TEF1 N-terminus tagged FAPoptimized</td>
<td>TEF1pr CEN LEU2</td>
<td>This study; FAP inserted at BamHI &amp; SmaI</td>
</tr>
<tr>
<td>pRS415-TEF1 C-terminus tagged FAPoptimized</td>
<td>TEF1pr CEN LEU2</td>
<td>This study; FAP inserted at SalI &amp; XhoI</td>
</tr>
<tr>
<td>pRS413-TEF1 N-terminus tagged FAPoptimized</td>
<td>TEF1pr CEN HIS3</td>
<td>This study; FAP inserted at BamHI &amp; SmaI</td>
</tr>
<tr>
<td>pRS413-TEF1 C-terminus tagged FAPoptimized</td>
<td>TEF1pr CEN HIS3</td>
<td>This study; FAP inserted at BamHI &amp; SmaI</td>
</tr>
<tr>
<td>pRS415-TEF1-Sec61- C-terminus tagged FAP original</td>
<td>TEF1pr-SEC61-FAP CEN LEU2</td>
<td>This study; FAP inserted at BamHI &amp; Sma; Sec61 inserted at BamHI &amp; HindIII</td>
</tr>
<tr>
<td>pRS415-TEF1-Sec61- C-terminus tagged FAP optimized</td>
<td>TEF1pr-SEC61-FAP CEN LEU2</td>
<td>This study; FAP inserted at BamHI &amp; Sma; Sec61 inserted at BamHI &amp; HindIII</td>
</tr>
<tr>
<td>pRS415-TEF1-Sec63- C-terminus tagged FAP original</td>
<td>TEF1pr-SEC63-FAP CEN LEU2</td>
<td>This study; FAP inserted at BamHI &amp; Sma; Sec63 inserted at BamHI &amp; HindIII</td>
</tr>
<tr>
<td>pRS415-TEF1-Sec63- C-terminus tagged FAP optimized</td>
<td>TEF1pr-SEC63-FAP CEN LEU2</td>
<td>This study; FAP inserted at BamHI &amp; Sma; Sec63 inserted at BamHI &amp; HindIII</td>
</tr>
<tr>
<td>pRS415-TEF1 N-terminus tagged FAP optimized</td>
<td>TEF1pr CEN LEU2</td>
<td>This study; FAP inserted at BamHI &amp; SmaI</td>
</tr>
<tr>
<td>pRS415-ADH1 N-terminus tagged FAP optimized</td>
<td>ADH1pr CEN LEU2</td>
<td>This study; FAP inserted at BamHI &amp; SmaI</td>
</tr>
<tr>
<td>pRS415-MET25 N-terminus tagged FAP optimized</td>
<td>MET25pr CEN LEU2</td>
<td>This study; FAP inserted at BamHI &amp; SmaI</td>
</tr>
<tr>
<td>Plasmid Name</td>
<td>Tagged Gene</td>
<td>CEN</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td>-----</td>
</tr>
<tr>
<td>pRS415-CUP1 N-terminus tagged FAP optimized</td>
<td>CUP1pr CEN LEU2</td>
<td>This study; FAP inserted at BamHI &amp; SmaI</td>
</tr>
<tr>
<td>pRS413-TEF</td>
<td>TEF1pr CEN HIS3</td>
<td>(Mumberg et al., 1995)</td>
</tr>
<tr>
<td>RS413-TEF1-ANP1-CFAoptmized</td>
<td>TEF1pr-ANP1-FAP CEN HIS3</td>
<td>This study; FAP inserted at SalI &amp; XhoI; Anp1 is placed between RE BamHI &amp; SmaI</td>
</tr>
<tr>
<td>RS413-TEF1-ERG6-CFAoptmized</td>
<td>TEF1pr-ERG6-FAP CEN HIS3</td>
<td>This study; FAP inserted at SalI &amp; XhoI; Erg6 inserted at BamHI &amp; SmaI</td>
</tr>
<tr>
<td>RS413-TEF1-PMA1-CFAoptmized</td>
<td>TEF1pr-PMA1-FAP CEN HIS3</td>
<td>This study; FAP inserted at SalI &amp; XhoI; Pma1 inserted at BamHI &amp; SmaI</td>
</tr>
<tr>
<td>RS413-TEF1-RPA34-CFAoptmized</td>
<td>TEF1pr-RPA34-FAP CEN HIS3</td>
<td>This study; FAP inserted at SalI &amp; XhoI; Rpa34 inserted at BamHI &amp; SmaI</td>
</tr>
<tr>
<td>RS413-TEF1-SEC7-CFAoptmized</td>
<td>TEF1pr-SEC7-FAP CEN HIS3</td>
<td>This study; FAP inserted at SalI &amp; XhoI; Sec7 inserted at BamHI &amp; SmaI</td>
</tr>
<tr>
<td>RS413-TEF1-VPH1-CFAoptmized</td>
<td>TEF1pr-VPH1-FAP CEN HIS3</td>
<td>This study; FAP inserted at SalI &amp; XhoI; Vph1 inserted at BamHI &amp; SmaI</td>
</tr>
</tbody>
</table>

4.2.3 **Fluorescence-activating protein (FAP) staining and confocal microscopy imaging**

To assess FAP-tag localization, yeast cells containing the indicated FAP-tagged plasmid were grown overnight to saturation in SC medium (pH 6.6) lacking the appropriate nutrient for selection unless otherwise indicated. Cells were then diluted to an A<sub>600</sub> of 0.3 into fresh medium and grown for and additional 4-5 h at 30°C, until cells reached mid-exponential phase growth (A<sub>600</sub> = 0.5-0.7). Cells were plated on 35-mm glass-bottom microwell dishes coated with poly-D-lysine (MatTek Corp., Ashland, MA) or coated with 15ul of 0.2mg/ul conc of concanavalin A (MP Biomedicals, Irvine, CA) and then either 1uM of MG-ESTER (cell-permeant dye called αRed-p, from SpectraGenetics, Pittsburgh, PA) or MG-B-TAU (cell impermeant dye called αRed-np, from SpectraGenetics, Pittsburgh, PA) was added, incubated for 10 min at room temperature and imaged immediately. Images were acquired with a Nikon Eclipse Ti inverted microscope outfitted with a Prairie-swept field confocal scan head (Nikon, Chiyoda, Tokyo, Japan; Prairie Instruments,
Middleton, WI), an Apo 100X objective (numerical aperture, 1.49), an Agilent monolithic laser launch (Agilent Technologies, Inc), and an Andor iXon3 camera (Oxford Instruments, Andor Technologies). NIS-Elements software (Nikon Instruments Inc.) was used to set imaging parameters, and all images within an experiment were captured using the identical settings. Fluorescence of MG-B-TAU and MG-ESTER dyes bound to FAP were excited using the 640-nm laser line and fluorescent emission was detected at 664 nm. For all images within an experiment the look-up-tables were adjusted equivalently in NIS-Elements software to generate figures that are representative of the primary data and the fluorescent intensity changes that were captured between samples.

4.2.4 Image quantification and statistical analysis

ImageJ software was used to quantify fluorescence intensity for all images. To quantify total fluorescence for both Mg-B-Tau and Mg-Ester dye cells were outlined manually in ImageJ. Mean pixel intensities were measured in arbitrary units (a.u.) for each cell, and the mean background pixel intensity was subtracted. The distribution of mean pixel intensities in a.u. and the distribution of pixel intensities for each group of cells are presented as scatter plots, where the horizontal midline represents the mean with the 95% confidence interval. A nonparametric Kruskal-Wallis test was performed using prism software (GraphPad, La Jolla, CA). Statistically significant differences were indicated in the figures by asterisks with the associated $p$ values provided in the figure legends.
4.2.5 Optimizing pH experiments

To identify an optimum pH, two methods were used. Yeast were grown in synthetic complete (SC) medium prepared as described (Johnston et al., 1977) and supplemented with 5M sodium hydroxide until a pH of 6.6 was reached. Alternatively, yeast were grown in Buffered Synthetic Media (BSM) [2% glucose, 5 mg/ml casamino acids, 1.7 mg/ml Yeast Nitrogen, 5.3 mg/ml (NH₄)₂SO₃, 2 ug/ml uracil, 100 mM Na-phosphate (pH 6.5)] as described in (Emmerstorfer-Augustin et al., 2018).

4.2.6 Flow cytometry

To quantitatively assess FAP fluorescence in dynamic populations, yeast cells containing the indicated FAP-tagged marker were grown overnight to saturation in SC medium at pH 6.6, re-inoculated to an A₆₀₀ of 0.3 in fresh medium and grown for 4-5 hours at 30°C until cells reached mid-exponential phase growth (A₆₀₀ = 0.5-0.7). Fluorescence was analyzed using the Attune Nxt Flow Cytometer (ThermoFisher Scientific). Flow rate was set to collect a total of 10,000 events at 100μl per second. The RL2 laser was used for excitation of the FAP detecting through a 720/30 filter. Data analysis was done using FlowJo software (Becton, Dickinson & Company, Franklin Lakes, NJ) to gate samples in forward scatter and side scatter to exclude non-yeast events and clumps of cells and obtain fluorescence values. RL2 fluorescence is shown.
4.2.7 Yeast protein extraction and immunoblot analysis

To analyze protein abundance, yeast whole cell protein extracts were made by growing cells in SC medium with appropriate nutrient selection to mid-exponential phase at 30°C (A600 of 0.5-0.7) then harvesting equivalent densities of cells by centrifugation (3 ODs of cells). Cell pellets were flash frozen in liquid nitrogen and stored at -80°C. To make extracts, pelleted cells were lysed and proteins precipitated using the trichloroacetic acid extraction method as described in (Volland et al., 1994a). Protein precipitates were solubilized in SDS/urea sample buffer (40mm Tris [pH 6.8], 0.1 mm EDTA, 5% SDS, 8M urea, and 1% β-mercaptoethanol) (O'Donnell et al., 2013) and heated to 37°C for 15 min prior to analyses by SDS-PAGE. Extracts were resolved by SDS-PAGE and proteins identified by immunoblotting. Either Revert™ 700 Total Protein stain (LI-COR BioSciences, Lincoln, NE) of the membranes or anti-Zwf1 antibody (MilliporeSigma, St. Louis, MO) was used as a loading and transfer control. Immunoblots were probed with mouse monoclonal anti-Myc (Thermo Scientific, Waltham, MA), rabbit polyclonal anti-RFP (Rockland Immunochemicals, Inc., Pottstown, PA), or rabbit polyclonal anti-Zwf1 (MilliporeSigma, St. Louis, MO) at the dilutions indicated from the manufacturers. Anti-mouse or anti-rabbit secondary antibodies conjugated to IRDye-800 or IRDye-680 (LI-COR BioSciences, Lincoln, NE) were detected using the Odyssey CLx infrared imaging system (LI-COR BioSciences, Lincoln, NE).
4.3 Results

4.3.1 Codon optimization of FAP for expression in yeast

The FAP technology employs a SCA that is genetically encoded as a fusion to the protein of interest. When the SCA binds a fluorogen dye it becomes fluorescent (Figure 20A) (Szent-Gyorgyi et al., 2008). The fluorogen can be derivatized to be cell permeant, in the MG-ester form, or cell impermeant, in the MG-B-tau form (Figure 20, B and C) (Szent-Gyorgyi et al., 2008; Szent-Gyorgyi et al., 2013). This latter version allows for imaging of the cell surface population of the protein of interest and is a useful tool for quantitative endocytic assays, but to date this approach has been used on a very limited scale in *S. cerevisiae* (Figure 20D) (Emmerstorfer-Augustin et al., 2018; Hager et al., 2018).

![Diagram of FAP technology](image)

*Figure 21. Utility of the fluorogen activating protein to visualize proteins of interest*
(A) When the SCA, that is fused to the protein of interest, binds the MG-derived fluorogen dye, fluorescence is detected. (B and C) The MG-derived dye can either be cell permeant (B) or cell-impermeant (C). (D) Use of the cell-impermeant dye allows for quantitative endocytic assays allowing for measurement of internalization rates.

To optimize the FAP sequence for expression in *Saccharomyces cerevisiae*, we used JAVA Codon Adaptation Tool (JCat) (Technical University of Braunschweig, Brunswick, Germany) (Grote et al., 2005). JCat employs the Codon Adaption Index (CAI) to generate an optimized sequence. CAI measures the codon usage bias, which is the frequency of occurrence of synonymous codons and their cognate tRNAs in the organism under study. The CAI number ranges from 0 to 1 for each codon, where values approaching 1 indicate a codon where the pairing tRNA is abundant in that organism. JCat then generates a new DNA sequence that encodes the same amino acids as the original sequence but ensures that the codons used for each amino acid are those with highly abundant cognate tRNAs. Before optimization, the FAP sequence had an average CAI of 0.063, indicating that most of the codons being used were matched to low abundance tRNAs in yeast. However, after optimizing the sequence using JCat the CAI value was 0.973, indicating the codon usage was now balanced to use more abundant tRNAs in yeast (Figure 21A). We sought to determine if this codon optimized version improved FAP function and/or expression in yeast. We ordered a gene block, which is synthesized base pairs, containing the codon optimized FAP sequence (Genewiz, South Plainfield, NJ) and tagged both the codon optimized FAP sequence and the original FAP sequence with a MYC epitope to facilitate biochemical detection. We further incorporated an up- or down-stream linker region (encoding 6 amino acids: Ala-Gly-Ala-Gly-Ala-Gly) to facilitate folding of the FAP and the protein to which it was fused (Figure 21B). Both the codon-optimized FAP (referred to as FAP\textsubscript{optim}) and the original FAP (referred to as FAP\textsubscript{origin}) were expressed under control of the *TEFI* promoter as soluble proteins or as fusions to the ER-resident translocon proteins, Sec61 and Sec63 (Stirling et al., 1992;
Young et al., 2001). We found there was not a significant increase in the fluorescence intensity of FAP\textsubscript{optim} compared to FAP\textsubscript{origin} when they were expressed as either free soluble proteins or when fused to Sec61 or Sec63 (Figure 21, C and D). Additionally, we used flow cytometry analyses to compare the fluorescence intensities for a larger population of cells and, consistent with the microscopy, the FAP\textsubscript{optim} signal was not different than the FAP\textsubscript{origin} signal (Figure 21, E-G). To further evaluate differences between FAP\textsubscript{optim} and FAP\textsubscript{origin}, we assessed FAP-tagged protein abundance via immunoblotting. There was significantly more soluble FAP\textsubscript{optim} present in cells than FAP\textsubscript{origin}, and although similar abundances were observed for FAP\textsubscript{optim} and FAP\textsubscript{origin} fusions to Sec61 and Sec63, we consistently observed breakdown products when FAP\textsubscript{origin} constructs were expressed (Figure 21, H and I). Taken together these findings suggest that proteins tagged with FAP\textsubscript{origin} may be more susceptible to degradation in yeast than FAP\textsubscript{optim}, making FAP\textsubscript{optim} better equipped for expression in yeast.
Figure 22. Codon optimization of the FAP tag

(A) Comparison of the codon adaptation index for each amino acid position in the FAP<sub>origin</sub> sequence (gray) versus FAP<sub>optim</sub> (purple). (B) Diagrams representing FAP constructs to be tagged at either the N- or C- terminus, each with a MYC epitope and a stream linker region that encodes six amino acids: Ala-Gly-Ala-Gly-Ala-Gly. (C) WT cells expressing either a soluble FAP<sub>origin</sub> or FAP<sub>optim</sub> or FAP<sub>origin</sub> or FAP<sub>optim</sub> fused to ER resident proteins Sec61 or Sec63. (D) The whole cell fluorescence intensity from n> 100 cells depicted in C were quantified and are presented as scatter
plots. The horizontal midline in each plot represents the mean fluorescence intensity, and error bars represent 95% CI. Kruskal-Wallis statistical analyses with Dunn’s post hoc test were performed, and the statistical significance is indicated (**, p<0.001; ***, p<0.0001; n.s., p>0.01, not significant). (E-G) Flow cytometry analysis comparing the fluorescence intensity of optimized FAP constructs versus original FAP constructs. These figures are representative of four replicates. Cells were stained with MG-Ester to acquire whole cell fluorescence. The mean is represented by the dotted lines. (H) Whole-cell extracts from WT cells expressing FAP<sub>origin</sub> or FAP<sub>optim</sub> were analyzed by immunoblotting with quantification, n=3. (I) Whole-cell extracts from WT cells containing indicated plasmids were analyzed via immunoblotting (α-MYC) and loading was controlled for via α-Zwf1. White dots indicate break down product of FAP<sub>origin</sub>. Molecular masses are denoted in kilodaltons.

**4.3.2 Imaging parameters that influence FAP fluorescence**

To achieve a robust imaging signal, we identified the parameters that modify FAP<sub>optim</sub> signal intensity. We first determined the effects of pH on FAP fluorescence intensity. It has been previously reported that the FAP<sub>origin</sub> signal is influenced by changes in pH (Emmerstorfer-Augustin et al., 2018). We hypothesized that pH would also affect FAP<sub>optim</sub> brightness since the amino acid sequence of FAP<sub>optim</sub>, and therefore its biochemical makeup, is the same as FAP<sub>origin</sub>. We expressed both soluble FAP<sub>optim</sub> and Sec61-FAP<sub>optim</sub> protein fusions under the <i>TEF1</i> promoter in cells grown in medium where the final pH had been adjusted (pH 5.8, 6.2, 6.6, 6.8, 7.0 or 7.2). We used two methods to alter the pH of the medium. First, as was done in Emmerstorfer-Augustin <i>et al.</i> (Figure 22 1, A-D), we used buffered synthetic media (see methods for recipe). Second we used synthetic medium with the addition of sodium hydroxide until the indicated pH was reached (Figure 22, A-D). With both soluble FAP<sub>optim</sub> and Sec61-FAP<sub>optim</sub> the fluorescence intensity was highest at pH 6.6 (Figure 22, A-D). Together, these data suggest that pH 6.6 gives superior fluorescent signal, but demonstrates that the FAP tag can be used under a range of pH conditions.
and still maintain its fluorescent properties, albeit with diminished overall signal. These data are consistent with previous work from Emmerstorfer-Augustin et al. which showed that pH 6.5 gave optimal fluorescence signal of FAP_{origin} (Emmerstorfer-Augustin et al., 2018).

**Figure 23. FAP fluorescence pH optimization**

(A & B) WT cells containing either soluble FAP or Sec61-FAP_{optim} imaged at a range of pH’s from 5.8 to 6.6. (B & C) Whole cell fluorescence intensities from the cells depicted in A or B were quantified via manual analysis.
using imageJ software and plotted as scatter plots. The horizontal midline in each plot represents the mean fluorescence intensity, and error bars represent 95% CI. Kruskal-Wallis statistical analyses with Dunn’s post hoc test were performed, and the statistical significance is indicated (**, *p*<0.001; ***, *p*<0.0001; n.s., *p*>0.01, not significant).

**Figure 24. Imaging parameters that influence FAP fluorescence**
(A & B) WT cells containing either soluble FAP or Sec61-FAPoptim imaged at a range of pH’s from 5.8 to 6.6. (B & C) Whole cell fluorescence intensities from the cells depicted in A or B were quantified via manual analysis using imageJ software and plotted as scatter plots. The horizontal midline in each plot represents the mean fluorescence intensity, and error bars represent 95% CI. Kruskal-Wallis statistical analyses with Dunn’s post hoc test were performed, and the statistical significance is indicated (**, p<0.001; ***, p<0.0001; n.s., p>0.01, not significant). (E) WT or pep4Δ cells containing either FAP-Ste3 or chromosomally integrated Ste3-GFP at its endogenous locus.

Next, we determined the best temperature to grow yeast strains expressing FAP-tagged proteins. To do this, we again employed soluble FAPoptim and Sec63-FAPoptim and compared fluorescence intensities in cells grown at 23°C versus those grown at 30°C. We found that the soluble FAPoptim signal was significantly brighter when grown at 23°C (Figure 23, A and C). However, when Sec63-FAPoptim was expressed, the fluorescence at either temperature was comparable (Figure 23, B and D). Thus, the optimal temperature for capturing fluorescence of a FAPoptim fusion protein is predicated upon the protein to which it is fused, and so should be assessed for each new fusion generated.
Figure 25. Optimal growth temperature for yeast for FAP imaging

(A & B) WT cells containing either soluble FAP or Sec61-FAP$_{\text{optin}}$ imaged at a range of pH’s from 5.8 to 6.6. (B & C) Whole cell fluorescence intensities from the cells depicted in A or B were quantified via manual analysis using imageJ software and plotted as scatter plots. The horizontal midline in each plot represents the mean fluorescence intensity, and error bars represent 95% CI. Kruskal-Wallis statistical analyses with Dunn’s post hoc test were performed, and the statistical significance is indicated (**, $p<0.001$; ***, $p<0.0001$; n.s., $p>0.01$, not significant).

4.3.3 FAP is degraded by a vacuolar protease

When assessing the localization of FAP-tagged membrane proteins it was apparent that there was almost no FAP signal in the vacuole for proteins under conditions in which trafficking to this compartment was well-established (Figure 12E). This is contrary to what we see with other fluorescent proteins such as GFP, which is a stable protein thus resulting in a robust signal in the vacuole (Reichard et al., 2016). The pH of the vacuole is ~6 in yeast, so we anticipated that FAP would continue to be fluorescent in this condition (Kane, 2006); however, imaging of membrane
proteins revealed that there was very little FAP fluorescence signal found in vacuoles in WT cells (Figure 23 E). We hypothesized that the FAP tag, unlike the stable β-barrel fold of GFP and other fluorescent probes (Chalfie et al., 1994), may not make a protease-resistant fold and so would be readily degraded in the vacuole and we would no longer accumulate fluorescence in this compartment. Alternatively, perhaps FAP-tagged proteins were not trafficking to the vacuole, making this reduced vacuolar fluorescence a critical feature to assess.

To determine if vacuole proteases were responsible for reduced fluorescence of FAP-tagged proteins reaching the vacuole, we assessed fluorescence of FAP-tagged Ste3 cells lacking Pep4, which is the protease that regulates maturation of most vacuole proteases, in comparison to wild-type cells (Ammerer et al., 1986). Ste3 is a G-protein coupled receptor involved in the yeast mating pathway. Ste3 resides on the cell surface of MATα cells and serves as the receptor for the a-factor pheromone secreted by MATa cells and this protein is known to undergo rapid constitutive endocytosis so that most of the fluorescence for a GFP-tagged version resides in the vacuole and often occludes detection of the surface population (Hagen et al., 1986). In pep4Δ cells vacuolar fluorescence for FAPoptim-Ste3 was observed and accumulated in the vacuole as was observed for Ste3-GFP (Figure 23E). Based on these observations, FAP-tagged Ste3 does traffic to the vacuole as expected, however unlike GFP it appears the FAP tag is degraded by the vacuolar proteases as it is only when Pep4 is deleted that we observed improved FAP fluorescent signal in this organelle. This was an exciting finding as it can be leveraged as an added useful facet of the FAP technology in yeast where trafficking of membrane proteins to the vacuole, and the subsequent bright fluorescence in this location, can interfere with quantification of other sub-cellular locales. Intermediates in the endosomes or Golgi can now be more readily observed when the fluorescent signal in the vacuole is quenched. This vacuolar quenching of FAP makes it similarly useful to
pHluorin, a variant of GFP that is unstable in the acidic environment of the vacuole (Prosser et al., 2016), but with the added adaptability of visualizing the vacuolar fluorescence if needed by deleting or inhibiting the vacuole proteases (*i.e.*, pepstatin treatment) (Umezawa et al., 1970; Wolff et al., 1996).

### 4.3.4 Development of tools with the FAP<sub>optim</sub> tag

Next, we generated a collection of FAP<sub>optim</sub>-tagging vectors where expression is driven from a range of promoters, each with distinct regulatory features (Figure 26A). The plasmids are based on pRS413 or pRS415 vectors (Christianson et al., 1992; Mumberg et al., 1995; Sikorski and Hieter, 1989), which contain the *HIS3* or *LEU2* selectable markers, respectively. We controlled expression of FAP<sub>optim</sub>-fusions using the following promoters: i) *TEF1*<sub>pr</sub>, for constitutive high-level expression (Mumberg et al., 1995), ii) *ADH1*<sub>pr</sub>, for modest-levels of constitutive expression (Mumberg et al., 1995), iii) *CUP1*<sub>pr</sub>, for copper-inducible expression (Labbe and Thiele, 1999) and iv) *MET25*<sub>pr</sub>, for methionine-repressible expression (gift from J.H. Hegemann, Univ. Düsseldorf (Figure 26A). For each promoter vector, we cloned the FAP<sub>optim</sub>-MYC sequence into two distinct locations within the multiple cloning site (MCS): 1) between *XhoI* and *SalI* to allow for proteins that would be tagged at their C-terminus with FAP<sub>optim</sub> or 2) between *BamHI* and *SmaI* for proteins that would be N-terminally tagged with FAP<sub>optim</sub>. A map providing the unique restriction sites for each of the plasmids is provided in Figure 26A. To assess FAP abundance and ensure FAP expression as anticipated, FAP<sub>optim</sub> was expressed as a soluble protein from each of the promoters in BY4741 yeast cells. The constitutive promoter *TEF1* and the repressible *MET25* promoter at 6 h post methionine removal gave similar FAP-fluorescence intensities and protein expression (Figure 26 B-D). As expected, based on prior studies, the
ADH1pr and CUP1pr at 120 min post copper-induction gave significantly lower levels of FAP-expression than TEF1 or MET25 promoters, as measured by fluorescence and protein abundance (Figure 26, B-D) (Labbe and Thiele, 1999; Mumberg et al., 1995). We also examined expression and fluorescence over a time-course of copper induction or methionine repression with the CUP1 and MET25 promoters, respectively, to determine the optimal timing for induction of soluble FAP_optim. We saw nice expression after two hours of methionine removal and after 90 minutes for copper addition (Figure 25 A-F). In sum, we generated 17 new cloning vectors for generating and expressing FAP-tagged fusion proteins under a wide range of conditions, allowing for maximal versatility for this system.
Figure 26. Assessing FAP-tag expression from various promoters

(A and B) Confocal microscope images of WT cells containing the indicated plasmids and incubated with methionine or copper to induce expression, MET25pr and CUP1pr respectively. (B and E) Total cellular fluorescence for n>100 cells, imaged as in B, was measured, and mean fluorescence intensities for all cells (in a.u.) are presented as scatter plots. The horizontal midline in each plot represents the mean fluorescence intensity, and the error bars represent the 95% confidence interval. Kruskal-Wallis statistical analyses with Dunn’s post hoc test were performed, and the statistical significance is indicated (**, p<0.001; p ***<0.0001; ns, p>0.01, not significant). (C and F) Whole cell extracts from cells expressing the indicated plasmids and incubated with methionine or copper to induce
expression, MET25pr and CUP1pr respectively, were resolved via SDS-PAGE and immunoblotted with anti-MYC antibody.

Figure 27. Generation of FAP-optim-tagging vector for expression of fusion proteins
(A) Plasmid map indicating different selection markers, promoters, and FAP placement available for the cell biology community. (B) Confocal microscope images of WT cells containing the indicated plasmids and grown to log phase (ADH1pr and TEF1pr) or incubated with methionine or copper to induce expression, MET25pr and CUP1pr respectively. (C) Total cellular fluorescence for n>100 cells, imaged as in B, was measured, and mean fluorescence intensities for all cells (in a.u.) are presented as scatter plots. The horizontal midline in each plot represents the mean fluorescence intensity, and the error bars represent the 95% confidence interval. Kruskal-Wallis statistical analyses with Dunn’s post hoc test were performed, and the statistical significance is indicated (**, p<0.001; p ***,<0.0001; ns, p>0.01, not significant). (D) Whole cell extracts from cells expressing the indicated plasmids were resolved via SDS-PAGE and immunoblotted with anti-MYC antibody.

4.3.5 FAP-tagged cellular markers as tools for co-localization studies in yeast

To encourage use of this FAP technology, we created a library of cellular markers tagged with FAP_{optim} for assistance in co-localization studies (Table 10). Since building these types of tools can be time consuming and sometimes challenging we wanted to create a toolkit that overcame this bottleneck. Each of these cellular markers were cloned into the pRS413-TEF1pr plasmid to ensure high expression (Figure 27A). We developed FAP_{optim}-tagged markers for the cis-Golgi (Anp1), lipid droplets (Erg6), the plasma membrane (Pma1), the nucleus (Rpa34), the trans-Golgi (Sec7), the endoplasmic reticulum (Sec61 and Sec63), and the vacuolar membrane (Vph1). To ensure correct localization, markers were co-expressed in cells where their respective endogenous protein was tagged with either RFP (Huh et al., 2003) or mNeonGreen (mNG) (constructed as in Materials and Methods section) and their co-localization assessed by fluorescence microscopy (Figure 27B). For Anp1, Erg6, Pma1, Rpa34, Sec61, and Vph1 we see colocalization of the FP-tagged version with endogenously tagged proteins. Additionally, these localizations are as expected based on the known cellular functions of these proteins in the Golgi,
lipid droplets, plasma membrane, nucleus, ER, and the limiting membrane of the vacuole, respectively (Figures 27, B and C). With these cellular markers there were not toxic effects on cell growth upon their expression.

Table 10. List of FAP-tagged cellular markers

<table>
<thead>
<tr>
<th>Protein</th>
<th>Organelle Location</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anp1</td>
<td>Golgi cis cisterna</td>
<td>Subunit of the alpha-1,6 mannosyltransferase complex</td>
</tr>
<tr>
<td>Erg6</td>
<td>ER, lipid droplets</td>
<td>Sterol 24-C-methyltransferase</td>
</tr>
<tr>
<td>Pma1</td>
<td>Plasma membrane</td>
<td>Plasma membrane P2-type H+ ATPase</td>
</tr>
<tr>
<td>Rpa34</td>
<td>Nuclear</td>
<td>RNA polymerase I subunit</td>
</tr>
<tr>
<td>Sec7</td>
<td>Trans-Golgi Network</td>
<td>Guanine nucleotide exchange factor (GEF) for ADP ribosylation factors</td>
</tr>
<tr>
<td>Sec61</td>
<td>ER</td>
<td>ER protein translocation channel</td>
</tr>
<tr>
<td>Vph1</td>
<td>Vacuole</td>
<td>Subunit of vacuolar-ATPASE V0 domain</td>
</tr>
</tbody>
</table>
Figure 28. FAP-tagged cellular markers

(A) Plasmid map illustrating vectors with FAP-tagged organelle markers incorporated. The FAP<sub>optim</sub>, equipped with two MYC tags at the 3’ end and an AGAGAG Linker at the 5’ end, was inserted between the restriction enzymes SalI and XhoI. Organelle markers were then C-terminally tagged via insertion between the restriction sites BamHI and Smal. (B and C) Co-localization was done via the co-expression of FAP-tagged markers with endogenously tagged protein: either RFP (B) or mNG (C). Anp1, a subunit of the alpha-1,6 mannosyltransferase complex, was tagged with FAP and co-localized with endogenously tagged Anp1-RFP. Erg6-FAP, a delta(24)-sterol C methyltransferase localized to lipid droplets, co-localizes with Erg6-RFP in lipid droplets as expected. The plasma
membrane P2-type H+-ATPase, Pma1, was tagged with FAP and localized at the plasma membrane with the Pma1-mNG. For a nuclear marker, we tagged Rpa34, an RNA polymerase I subunit found in the nucleus and we see that Rpa34-FAP co-localizes nicely with Rpa24-mNG. FAP-tagged Sec7, a guanine nucleotide exchange factor (GEF), co-localizes with endogenously tagged Sec7-mNG. Sec61, an ER translocation channel, tagged with FAP co-localizes to the ER along with endogenously tagged Sec61-mNG. We tagged the subunit of the vacuolar-ATPase V0 domain, Vph1, which localizes to the vacuolar membrane and can be seen co-localizing with endogenously tagged Vph1-mNG.

4.4 Discussion

Here we report the construction of a codon-optimized version of the fluorogen-activating protein (FAP) for expression in yeast, FAP\textsubscript{optim}. Although originally developed in yeast, the original FAP sequence was not codon optimized for expression in yeast and we find that the new codon optimized FAP we made here is expressed more robustly and degraded less. We further assessed the growth and pH conditions for optimal expression of FAP and, importantly, found that the FAP tag is susceptible to cleavage in the yeast vacuole. This contrasts with many FP, including GFP and mCherry, which are folded in such a way as to be resistant to vacuole protease digestion. This adds a novel dimension to the FAP\textsubscript{optim} tag as the fluorescence for this probe, much like that of pHluorin, is lost in the lumen of the vacuole. For protein trafficking studies, this is particularly useful as it eliminates the accumulation of bright fluorescence in the vacuole that can obscure fluorescent signals at other cellular locations. For studies where observing vacuolar fluorescence for the probe becomes important, this too can be achieved through genetic or chemical inhibition of the vacuole proteases.

We then generated a suite of cloning vectors and a series of intracellular markers using FAP\textsubscript{optim} to aid in protein trafficking and other studies. The addition of a far-red fluorescent probe
for live cell imaging can add a new dimension for co-localization studies and research aimed at the dissection of intracellular sorting pathways where scientists already utilize fluorescent proteins in the red, green, and blue channels. Excitingly, the FAP technology also allows for selective labeling at the cell surface. For example, fusing the SCA to the extracellular portion of the protein, as we have done with Ste3, allows for selective imaging of the population at the PM and thereby facilitates quantitative endocytic assays.

During plasmid construction, it was imperative to think about the placement of the FAP tag. In order to effectively use the impermeant dye for trafficking experiments, the FAP must be available at the exterior of the cell. For example, with Ste3 we have fused the FAP to the extracellular N-terminal, thus allowing the dye to interact. Additionally, to ensure correct localization we included a secretion signal to ensure that membrane proteins can enter the secretory pathway effectively as was done with the pheromone receptor Ste2 (Emmerstorfer-Augustin et al., 2018).

Additionally, we generated seven FAP-tagged markers for cellular compartments. We have demonstrated that each of these constructs accurately reflects the cellular locations they were meant to target by assessing their location in strains where the respective protein was chromosomally tagged with RFP or mNeonGreen (mNG) (Figure 27). While the most apparent benefits to using FAP lie in protein trafficking, the usefulness of this tag extends to co-localization studies. For example, the FAP-tag allows for the ability to induce the fluorescence signal after imaging other proteins, fully eliminating any issues with background fluorescence or possible fluorescent bleed-through when the target protein of interest is low abundance. FAP uses the far-red channel, which is not routinely used for live-cell imaging in yeast. With only a limited number of probes in this channel, it does not overlap spectrally with other commonly used fluorophores.
(i.e., GFP, YFP, RFP, BFP, etc.) making this an excellent probe to augment the existing fluorescent toolbox and facilitate multi-color imaging. Together, this work will give yeast biologists the tools they need to complete protein trafficking studies in this advantageous model organism.
5.0 Conclusions and Future Directions

I now summarize how this research advances the understanding of α-arrestin-mediated trafficking of the mammalian potassium channel Kir2.1 and how the tools I have created that incorporate FAP-tagging technology will benefit the scientific community. Additionally, I discuss potential experiments that would keep this research moving forward.

5.1 Significance of This Study

5.1.1 α-Arrestin-mediated trafficking of Kir2.1

The KCNJ family encodes inward-rectifying K⁺ channels known as KIRs (Hibino et al., 2010). KIR mutations cause disease, including cardiomyopathies, neurological and metabolic disorders (de Boer et al., 2010; Pattnaik et al., 2012). This work has focused on Kir2.1, which is expressed in the heart, muscle and neurons (Hibino et al., 2010). In short, Kir2.1 contributes to the K⁺ influx in cardiomyocytes after hyperpolarization, which allows cells to restore to resting potential (Houtman et al., 2014). Mutations in Kir2.1 lead to heart disease, with 21 disease-causing mutations found thus far (Donaldson et al., 2004). Specifically, loss-of-function mutations, which can stem from trafficking defects or K⁺ import malfunction, increase the QT interval in EKGs, causing Andersen-Tawil Syndrome (ATS) (Miake et al., 2003; Priori et al., 2005; Xia et al., 2005; Zaritsky et al., 2001). ATS patients suffer from a myriad of symptoms including cardiac arrhythmias, periodic paralysis, and dysmorphic skeletal features (Kimura et al., 2012). Several of
these mutations that disrupt Kir2.1 are linked to defective protein trafficking to the PM (Ballester et al., 2006; Ma et al., 2011; Taneja et al., 2018). For example, the ATS-causing mutation Kir2.1\(^{Δ314-315}\) reduces Kir2.1 at the PM by preventing binding to clathrin adaptor complex, AP-1 (Ma et al., 2011). Conversely, retrograde Kir2.1 sorting in the Golgi is regulated by the Golgin-97 tether. Loss of Golgin-97 reduces Kir2.1 at the PM and its inhibition is a proposed therapeutic strategy for Kir2.1-linked diseases (Taneja et al., 2018; Zangerl-Plessl and van der Heyden, 2018). Although 60% of ATS cases are associated with a mutation in Kir2.1, in 40% of ATS cases the underlying mutation is not known leading room for improvement (Donaldson et al., 2004).

To advance our understanding of the molecular determinants that control Kir2.1 trafficking, we used a ‘humanized’ yeast model in both Chapter 2 and Chapter 3 where the endogenous yeast potassium channels, Trk1 and Trk2, were replaced with a mammalian potassium channel, Kir2.1. Yeast is an important model organism that has a history of being leveraged to study human channels such as the epithelium Na\(^+\) channel and Na\(^+-\)phosphate cotransporter in addition to Kir2.1 (Buck et al., 2010; Dunham and Fowler, 2013; Kolb et al., 2011; Paddon et al., 1996). In this model system, yeast K\(^+\) channels were deleted so they fail to grow in low K\(^+\) medium. We then express mammalian Kir2.1, which forms a functional channel that localizes to the PM, imports K\(^+\) and rescues growth on low K\(^+\)-medium (Hager et al., 2018; Kolb et al., 2014). As highlighted above, this system has been successfully used to identified Kir2.1 regulators by Kolb et al. (Kolb et al., 2014). Of interest to us is that in a genome wide yeast deletion screen (~4000 single gene deletions), nearly 2/3 of the genes that improved growth on low K\(^+\) medium were in protein trafficking (Kolb et al., 2014). We then performed a targeted screen to determine if the α-arrestins, the protein trafficking adaptors studied extensively in our lab, influenced Kir2.1 PM residency. Excitingly, we found that three α-arrestins–Aly1, Aly2, and Ldb19–regulate Kir2.1
trafficking to the PM as highlighted in Chapter 2 (Hager et al., 2018). Furthermore, in identifying these α-arrestins as important for Kir2.1 trafficking, we assessed how they are regulated. To this end, we found that this α-arrestin-mediated trafficking was dependent on the ubiquitin ligase Rsp5, and the protein phosphatase calcineurin (see Chapter 2).

Next we set out to identify the trafficking machinery used by the α-arrestins to traffic Kir2.1 using a similar approach with serial dilution assays. Specifically, we deleted key trafficking machinery we hypothesized to be involved and assessed the ability of the α-arrestins to rescue growth on low potassium medium. From this work we identified the AP-1 complex, AP-2 complex, ESCRT complex, and retromer complex as well as Vrp1 and protein kinase, Npr1, as being essential for select α-arrestin-mediated trafficking of Kir2.1 (Chapter 3).

5.1.2 Development of the fluorescent activating protein for yeast trafficking studies

The use of fluorescent proteins has been vital to trafficking studies. These probes allowed scientists to better visualize and thus understand cell biology and protein trafficking. Among the many advancements in this field, are the second generation of biconjugate tags. Of special interest to us is the fluorogen activating proteins (FAPs) that we outlined in Chapter 4. I first used the FAP protein technology out of necessity to study the protein trafficking of Kir2.1. Specifically, in order to more accurately study Kir2.1 protein trafficking, a system that differentiated between protein at the plasma membrane and the bright ER-retained pool of Kir2.1 was required. Before this work, FAP technology had scarcely been used in mammalian cell systems (Lorenz-Guertin et al., 2017). Previous methods of fluorescent imaging were unable to discriminate between these two pools of proteins. However, certain characteristics of the FAP technique allowed us to discern between the
two populations. Specifically, this technology has two components; a single chain antibody (SCA) that can be fused to a protein of interest and fluorogens, which are dyes that are non-fluorescent when free in solution. The choice between a membrane-permeant dye and a nonmembrane-permeant dye allows for cell surface fluorescence only or whole cell fluorescence, thus allowing for the ability to differentiate between the two Kir2.1 populations. This technology aided in the identification of the α-arrestins as regulators of Kir2.1 trafficking (Hager et al., 2018). As this was the first time this technique was used in a yeast model, it paved the way for future yeast studies to also take advantage of this technique. To help ease that process, I created a series of FAP-tagged vectors that would simplify the construction process when others go to FAP-tag their protein of interest as well as create cellular markers to help aid in co-localization studies for live cell imaging. Furthermore, I optimized imaging conditions as well as the FAP sequence itself for the yeast model organism (Chapter 4).

5.1.3 Limitations of this study

While the ‘humanized’ yeast system for studying Kir2.1 trafficking is powerful in that it allows us to genetically modify the trafficking framework at will, there are limitations to this tool. For example, when we assess the ability of Kir2.1 to rescue growth on low potassium medium in the presence of a gene delete the growth readout straight-forward, but it does not account for compensatory changes that could arise because of that gene deletion. When strictly interpreted through the lens of altering Kir2.1-dependent trafficking, there may be facets that are missed that arise from changes in Kir2.1 protein abundance or from altered expression of some other trafficking factor in this cellular background. Further, when we also express the α-arrestin in the gene deletion backgrounds, this adds a layer of complication to the readout. Are the α-arrestins
themselves equally expressed or similarly localized in these new gene deletion backgrounds? We have not yet assessed this facet and it could influence regulation of Kir2.1 dramatically. In addition, in some gene deletion backgrounds there is very robust growth on low potassium medium, and this makes it impossible to assess the impact of α-arrestin on trafficking/function of Kir2.1. For example, as we see with trk1Δ trk2Δ end3Δ, we cannot assess α-arrestin’s ability to improve growth as loss of end3 already results in robust growth on the lowest KCl concentration. Furthermore, this system is currently dependent on plasmid born expression of both Kir2.1 and the α-arrestins. Although we select for plasmids by manipulating media conditions, it may be difficult for strains, especially those that grow poorly, to express both Kir2.1 and the α-arrestin. In addition, plasmid driven expression gives rise to higher degrees of cell-to-cell variability. In future, a system where Kir2.1-FAP is integrated in the genome and expressed from an inducible promoter system may make these studies more robust. Similarly, to see effect of the α-arrestins, we must overexpress them, which also leads to added variability. In addition, the fact that Kir2.1 predominately resides in the ER in the yeast model system also impacts these studies. It makes it difficult to see the pool of Kir2.1 out at the PM even with confocal microscopy and the selectivity of the FAP tag. In addition, it provides a constant source of Kir2.1 that, should trafficking and folding permit, can be mobilized to alter the cell surface abundance of Kir2.1. The next step in this project will be to apply this technology to a mammalian model system. Although there are mammalian α-arrestins, it is difficult to determine which mammalian α-arrestins are the homologs of the yeast α-arrestins.
5.2 Future Directions

The future of our α-arrestin-mediated protein trafficking of Kir2.1 work should focus on uncovering the relationship between the mammalian α-arrestins and Kir2.1 in HEK293T cells and primary cardiomyocytes. Preliminary studies have been started and already yield exciting results (Appendix A). If I were able to continue this work or guide a new student on this project I would suggest the experiments below.

5.2.1 Short term

Our preliminary work in the yeast model system indicates that ArrDC2 and ArrDC3 are likely to control Kir2.1 trafficking. Thus, the first steps I would take moving forward would be to complete the experiments outlined in Chapter 2, but with ArrDC2 and ArrDC3 in the yeast model system. Specifically, this includes confirming the rescued growth phenotype that we see in Appendix A (Figure 28A). Using the FAP technology that is highlighted in Chapter 2 and Chapter 4 to measure any localization changes ArrDC2 or ArrDC3 may cause. Ultimately, demonstrating that these mammalian α-arrestins also promote the Kir2.1 localization to the PM, as occurs with yeast α-arrestins, would be validation of the yeast model and add new regulatory factors to the control of Kir2.1 (Figure 4 and Figure 9). If there is a similar result then this would be great evidence that what we see with yeast α-arrestins is conserved to the mammalian α-arrestins. In parallel to these experiments, using ICP-MS to measure the intracellular levels of potassium, again to determine if the expression of ArrDC2 and ArrDC3 increases these levels as we see with yeast α-arrestins (Figure 4). In addition to studies that are done in Chapter 2, it would be interesting to
test the direct interaction between the mammalian α-arrestins and Kir2.1. This would involve creating a library of site-directed Kir2.1 mutants in N- and C- terminal tail and then testing the ability of the α-arrestins to rescue growth on low potassium medium. Here I think it would be easy enough to test both the yeast α-arrestins and the mammalian α-arrestins. If there are “hits” from this experiment, then I would suggest recreating the hits in the FAP-Kir2.1 system and analyzing their ability to localize Kir2.1 to the PM. In HEK293T cells, co-purification assays would further help determine the direct relationship between mammalian α-arrestins and Kir2.1. Additionally, mutants of interest from the previously mentioned experiments should be used here to narrow down the point of interactions.

With our yeast model system, it has become quite clear the importance that ubiquitin plays when it comes to regulating the α-arrestins (Lin et al., 2008; Nikko and Pelham, 2009; O'Donnell et al., 2010). Could this be true for the mammalian α-arrestins as well? In order to do this, the PY-motifs of the mammalian arrestins need to be mutated, similarly to our PPXY-less mutants in Chapter 2 (Hager et al., 2018). Once these are generated, I would suggest completing the same experiments as stated above. In short, I would assess their ability to rescue growth on low potassium medium, traffic Kir2.1 to the PM, raise intracellular potassium levels, and ability to interact with Kir2.1. Using these same mutants in mammalian cells, I would again assess their effect on Kir2.1 localization and also abundance. Furthermore, the ubiquitin ligase in mammalian cells is unknown. To identify the unknown ubiquitin ligase co-purification studies or MS analysis could be done (O'Donnell et al., 2010).

Furthermore, there is much to still be discovered in our yeast model system when it comes to trafficking factors. Although I would focus on transitioning to mammalian studies, there are several pathways, specifically recycling pathways, that deserve attention. For example, there is
very recent work that discusses the regulation Ist1 has over the recycling of nutrient permeases (Kamilla et al., BioRxiv). This work provides evidence that Ist1 is involved in endosome recruitment by promoting recycling. Deletion of this gene in our trk1 trk2Δ yeast model would further shed light on the pathway the α-arrestins use to traffic Kir2.1. It also would be interesting to see if Ist1 alone is a regulator of Kir2.1. I would expect upon deletion of Ist1 we would see a decrease of growth on low potassium in a Kir2.1-dependent manner and I hypothesize that this deletion would either hinder the ability of Aly2 to still increase growth on low potassium, or if Aly2 works through another mechanism, its ability to improve growth would be unaffected.

5.2.2 Long Term

First, a clear regulatory tie between mammalian α-arrestins and Kir2.1 needs to be forged in cell lines, which will make assessment of associations between α-arrestins and Kir2.1 easier. Once this is completed, I would move to studies of mammalian α-arrestins in cardiomyocytes to determine if they regulate Kir2.1 trafficking and function in the heart. From my work in cardiomyocytes, it is clear that making use of adenoviral infection to overexpress mammalian α-arrestins is the best route for studying the impact of these adaptors on Kir2.1 (Merkel et al., 2019). When just doing transient transfections with plasmids expressing α-arrestins, there is very low transfection rate in cardiomyocytes (~8%) (Djurovic et al., 2004). Switching to an adenoviral expression system will give a far greater number of cardiomyocytes with plasmid expression (Djurovic et al., 2004). For example, in some transfections I was only able to identify three cells expressing the α-arrestins. Once the viral expression system is in place, I would suggest conducting several imaging experiments. First, live-cell imaging to see where the α-arrestins
themselves localize with in the cell. During these experiments, I would suggest capturing a set time frame in order to analyze the movement of the α-arrestins throughout the cell. Additionally, it would be beneficial to use adenoviral infection to also express a fluorescently tagged Kir2.1. This would allow for visualizing both Kir2.1 and the α-arrestins during live cell imaging, which could give great insight in the relationship between the two proteins. In addition to live cell imaging, I suggest using immunofluorescence microscopy. Here I think it will be extremely important to utilize cellular markers or stains. In order to understand the protein trafficking relationship between the α-arrestins and Kir2.1, it will become critical to identify the organelles or trafficking machinery also involved in this relationship. Once there is confirmation of interaction and/or co-localization between the α-arrestins and Kir2.1, I would broaden focus to see what other cellular components are involved. For example, do these proteins use clathrin-mediated endocytosis to traffic throughout the cell? In order to do this, endocytic inhibitors can be used and the affect they have on the α-arrestin-mediated trafficking of Kir2.1 can be analyzed. The next step to studying this relationship would be to knockdown machinery we suspect is essential for this trafficking pathway. This is a similar approach that we used in Chapter 3, however, a bit more difficult in this system. In order to achieve this interfering RNAs can be used to knockdown different machinery. On this note, it would also be interesting to knockdown each of the α-arrestins and see the affect this has on the localization of Kir2.1. In a series of experiments where I assessed Kir2.1 localization in a 9 arrestin delete background, wherein which 9 of the 14 α-arrestins are deleted, Kir2.1 localization at the PM dramatically increased, which is a result I would expect to see if the α-arrestins were involved in the endocytosis of Kir2.1, not the recycling. Furthermore, it would be very interesting to analyze what the relationship between the α-arrestin and Kir2.1 are in mammalian cells. We have strong evidence (Chapter 2) that the yeast α-arrestins are involved
in recycling of Kir2.1, but this is in a very different model organism so it would be of importance
to define what the relationship is in primary cells. Additionally, in our yeast model system we have
begun to identify the pathway used by the α-arrestins. Many of these complexes outlined in
Chapter 3 such as AP-1, retromer, and ESCRT are conserved in mammalian cells. This would be
a good starting out place for RNA knockdown studies.
Appendix A Exploring the Regulation of Kir2.1 By Mammalian α-Arrestins

This project was developed in collaboration with Dr. Adam V. Kwiatkowski in the Department of Cell Biology, School of Medicine, University of Pittsburgh. All experiments in primary cardiomyocytes were conducted in the Kwiatkowski lab by myself, but with guidance from Dr. Kwiatkowski. This project is ongoing, but data shown represents work that I completed during my degree. Experiments done in HEK293T cells were done with guidance from Dr. Annette Chiang.

Appendix A.1 Introduction

With every heartbeat, potassium ions rush out of and calcium ions rush into cardiomyocytes to drive heart contraction. Ion balance is restored after contraction, in part, by the inward rectifying K⁺ channel Kir2.1. Specifically, Kir2.1 controls the influx of potassium into cardiomyocytes after hyperpolarization, restoring the cell to its initial resting potential and setting the stage for the next action potential (Houtman et al., 2014). Mutations in Kir2.1 lead to heart disease, with 21 disease-causing mutations identified (Donaldson et al., 2004); Gain-of-function mutations shorten the QT interval in electrocardiogram (EKG) readings, causing cardiac arrhythmia and atrial fibrillations due to loss of hyperpolarization (Houtman et al., 2014), while loss-of-function mutations lengthen the QT interval in EKG readings and result in Andersen-Twail Syndrome (ATS). ATS patients suffer from cardiac arrhythmias, periodic paralysis, and dysmorphic skeletal features such as cleft lip (Kimura et al., 2012). Mutations that disrupt Kir2.1
function are often linked with defective delivery to the plasma membrane and thereby altered Kir2.1 function (Ma et al., 2011). Trafficking of Kir2.1 to the plasma membrane is essential for cardiac function, yet little is known about the molecular mechanisms underlying Kir2.1 sorting to and from the plasma membrane. To shed light on this trafficking, we used “humanized” yeast model system – where cells depend on ectopically expressed Kir2.1 – to define Kir2.1 trafficking regulators. Excitingly, we found that a family of trafficking adaptors, known as the α-arrestins, regulate Kir2.1 trafficking to the plasma membrane (See Chapter 2).

To date, little is known about the mammalian α-arrestins (called ArrDC1-5 and TXNIP), but they regulate membrane protein trafficking and thus are well suited to regulate Kir2.1. To begin investigating the role of the mammalian α-arrestins, we employed our “humanized” yeast cell system to define the trafficking of Kir2.1. In this yeast model, Kir2.1 is ectopically expressed in cells where the endogenous potassium channels, TRK1 and TRK2, are deleted causing sensitivity to growth on low potassium medium. Expression of Kir2.1 in this system modestly improves growth of trk1Δ trk2Δ cells on low potassium medium, demonstrating that the channel functions to bring potassium into the cells (Kolb et al., 2014). This system has been successfully used to identify components that are conserved to mammalian models. Specifically, the subunits in the ESCRT complex, which are needed to form and sort proteins into multivesicular bodies, were identified as critical regulators of Kir2.1 trafficking in yeast and mammals (Kolb et al., 2014). Using this yeast model system, we found that select α-arrestin – Aly1, Aly2, and Ldb10 – increase Kir2.1-dependent growth, plasma membrane localization, and intracellular K⁺ levels (See Chapter 2) (Hager et al., 2018). Based on these findings, we hypothesize that the mammalian α-arrestins will analogously regulate Kir2.1 trafficking.
Appendix A.2 Materials and Methods

Yeast strains and growth conditions

Yeast were grown in synthetic complete medium prepared as described (Johnston et al., 1977). SC low-potassium medium was also prepared as described with the use of mono-sodium glutamate as a nitrogen source, the addition of 20 mM MES to maintain the pH, and the indicated amount of KCl (Kolb et al., 2014). Liquid medium was filter-sterilized, and for plated medium, 2% (w/v) agar was added prior to autoclaving. Plasmids were transformed into yeast via the lithium-acetate method and selected for on the appropriate SC medium. Yeast cells were grown at 30°C. For growth assays on solid medium, 5-fold serial dilutions of saturated, overnight cultures were plated onto indicated medium and grown for 3-6 days.

Cardiomyocyte isolation and culture

This work was approved by the University of Pittsburgh Division of Laboratory Animal Resources under the oversight of Dr. Adam Kwiatkowski.

Neonatal mouse cardiomyocytes were isolated as described (Ehler et al., 2013)(Merkel et al., 2019). In short, mouse pups were killed between P1-P3, heart were then removed, cleaned, minced, and digested overnight at 4°C in 20 mM BDM (2,3-butanedione monoxime) and 0.0125% trpsin in Hank’s balanced salt solution. On day two, heart tissue was further digested in 15 mg/ml Collagenase/Dispase (Roche) in Leibovitz media with 20 mM DBM to create a single-cell suspension. Cells were preplated for 1.5-5 hours in plating media (65% high glucose DMEM, 19% M-199, 10% horse serum, 5% fetal bovine serum, and 1% penicillin-streptomycin) in order to remove endothelial cells and fibroblasts. Cardiomyocytes were then plated on MatTek dishes (1.5 x 10^5) in plating media. Sixteen hours after plating, the plating media was exchanged for
maintenance media (78% high glucose DMEM, 17% M-199, 4% horse serum, 1% penicillin-streptomycin, 1 µM AraC, and 1 µM Isoproterenol). Cells are now ready for transfections

Transfections in HEK293T cell and cardiomyocytes

HEK293T cells were plated 1x10^6 in 6 well dishes and grown overnight. HEK293T cells and cardiomyocytes (see above section) were then transiently transfected with the indicated plasmid(s) using Lipofectamine 2000 (Invitrogen). DNA was diluted in Opti-MEM medium (Thermo Fisher) and Lipofectamine 2000 was added to Opti-mem and both allowed to incubate for 5 min. These are then combined and allowed to incubate for 20 minutes then added to the cells. After 3 h the cells are given fresh media and grown overnight.

Immunofluorescence

Media was replaced with warm (37°C) PHEM fix [60 mM PIPES pH 7, 25 mM HEPES pH 7.0, 10 mM EGTA pH 8.0, 2 mM MgCl₂, 0.12M Sucrose, 4% Paraformaldehyde]. Cells were washed 2X in PBS + 0.02% azide. Once fixed, cells were permeabilized with PBS + 0.02% Triton X-100 for 2 min (HEK293T) or 4 min (cardiomyocytes) at RT. 2X wash in PBS. Primary antibody staining: Anti-Kir2.1 KCNJ2 (Allomone Labs, Jerusalem, Israel) was diluted at 1:100 in PBS + 1% BSA and stained for 1 hour at RT. Secondary antibody staining: α-RAT (480 nm) and α-phalloidin (Invitrogen, Carlsbad, CA) (647nm) were diluted 1:200 in PBS + 1% BSA and stained for 45 min at RT. Cells were washed 2X in PBS. Mounting media (Invitrogen, Carlsbad, CA) was added and coverslip placed on top. Let cure for 24 hours then imaged.
**Confocal microscopy**

Images were acquired using a Nikon Eclipse Ti inverted microscope outfitted with a Prairie-swept field confocal scan head, an Agilent monolithic laser launch, and an Andor iXon3 camera. NIS-Elements software was used to control the imaging parameters and all images within an experiment were captured using identical settings.

**Plasmids used in this study**

Plasmids used in this study and details of their construction are described in Table 11.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCDNA-ArrDC1-mCherry</td>
<td>Alvarez lab (Shea et al., 2012)</td>
</tr>
<tr>
<td>pCDNA-ArrDC2-mCherry</td>
<td>Alvarez lab (Shea et al., 2012)</td>
</tr>
<tr>
<td>pCDNA-ArrDC3-mCherry</td>
<td>Alvarez lab (Shea et al., 2012)</td>
</tr>
<tr>
<td>pCDNA-ArrDC4-mCherry</td>
<td>Alvarez lab (Shea et al., 2012)</td>
</tr>
<tr>
<td>pCDNA-TXNIP-mCherry</td>
<td>Alvarez lab (Shea et al., 2012)</td>
</tr>
<tr>
<td>pCDNA3.1-Kir2.1</td>
<td>(Kolb et al., 2014)</td>
</tr>
<tr>
<td>pRS415-TEF1pr-Kir2.1-HA</td>
<td>(Kolb et al., 2014)</td>
</tr>
<tr>
<td>pRS416-GPD-ArrDC1-mCherry</td>
<td>O’Donnell lab</td>
</tr>
<tr>
<td>pRS416-GPD-ArrDC2-mCherry</td>
<td>O’Donnell lab</td>
</tr>
<tr>
<td>pRS416-GPD-ArrDC3-mCherry</td>
<td>O’Donnell lab</td>
</tr>
<tr>
<td>pRS416-GPD-ArrDC4-mCherry</td>
<td>O’Donnell lab</td>
</tr>
<tr>
<td>pRS416-GPD-TXNIP-mCherry</td>
<td>O’Donnell lab</td>
</tr>
</tbody>
</table>
Appendix A.3 Results and Discussion

To assess the ability of the mammalian α-arrestins to regulate Kir2.1 trafficking, we employed our “humanized” yeast system, but this time we over-expressed the mCherry-tagged mammalian α-arrestins – ArrDC1, ArrDC2, ArrDC3, ArrDC4, and TXNIP – and assessed growth on low K+ medium (Figure 28A). We found that each of the mammalian α-arrestins was able to confer increased growth on low K+ media, when Kir2.1 was expressed but that ArrDC2 and ArrDC3 were able to more robustly rescue growth on low K+ medium (10 mM KCl). These findings are similar to what we have observed for over-expression of yeast α-arrestins (Hager et al., 2018) and suggest that they too might promote Kir2.1 localization to the plasma membrane in a similar manner to the select yeast α-arrestins (Figure 28A).
(A) Growth of serial dilutions of trk1Δ trk2Δ cells containing the indicated plasmids on SC medium lacking uracil and leucine and containing the indicated added concentrations of KCl is shown after 2 days of incubation. (B) Whole-cell extracts from WT cells containing mCherry-tagged α-arrestins alone or with Kir2.1 were analyzed via immunoblotting (α-HA, Kir2.1: 800; α-RFP, mCherry-tagged α-arrestins: 700) and loading was controlled for via Li-Cor total protein strain.

Using these same strains, we conducted analysis via western blotting. It appears Kir2.1 levels, see black arrow, remain unchanged with the addition of the α-arrestins (Figure 28B), and this is consistent with data we have shown before where the yeast α-arrestins do not change the overall abundance of Kir2.1, but instead alter the abundance of Kir2.1 at the PM (Hager et al., 2018). With most of the Kir2.1 expressed in yeast being retained in internal compartments (~90% retained in ER), it makes sense that we would not observe dramatic alterations in the overall abundance using this approach.

Figure 30. α-Arrestins in HEK293T cells
(A) Confocal imaging of fixed immunofluorescence stained HEK293T cells (RFP-tagged α-arrestins 532? excitation; α-HA Kir2.1 at 480 excitation; α-phalloidin at excitation 640).

We assessed Kir2.1 localization when α-arrestins are over-expressed in HEK293T cells. HA-tagged Kir2.1 and mCherry-tagged mammalian α-arrestins (ArrDC1, ArrDC2, ArrDC3, ArrDC4, and TXNIP) were transiently transfected into in HEK293T cells and their localization was assessed by immunofluorescence (IF) (Figure 29) Many α-arrestins localize to the plasma membrane and intracellular vesicles, whereas TXNIP was localized to the nucleus. In WT vector cells, Kir2.1 appears to localize to the PM. We see partial co-localization of ArrDC1, ArrDC2, ArrDC3, ArrDC4 with Kir2.1, and have the strongest co-localization between ArrDC1 and Kir2.1. Although we see no Kir2.1 co-localization with TXNIP, there appears to be increased Kir2.1 PM localization and reduced Kir2.1 puncta, suggesting that TXNIP also influences Kir2.1 localization, but differently than the other α-arrestins.
Figure 31. α-Arrestins in cardiomyocytes

Confocal imaging of fixed immunofluorescence-stained neonatal mice cardiomyocytes cells (RFP-tagged α-arrestins excitation; a-HA Kir2.1 at 480 excitation; α-phalloidin at excitation 640).

In wildtype cardiomyocytes, Kir2.1 localizes to the PM. When we expressed each of the mammalian α-arrestin in cardiomyocytes that were harvested from neonatal mice, preliminary results showed that Kir2.1 co-localized with ArrDC1, ArrDC2, and ArrDC3 (Figure 30). Again, these results are consistent with what we see in our yeast model system, where ArrDC1 and ArrDC3 improved Kir2.1-dependent growth on low K+ medium.
Thus, together these results are promising in that what we have found in our yeast model system will likely be conserved in a mammalian system. In summary, this work in mammalian cells is promising in that what we see in our yeast model system holds true in primary cells.


Ayscough, K.R., Stryker, J., Pokala, N., Sanders, M., Crews, P., and Drubin, D.G. (1997). High rates of actin filament turnover in budding yeast and roles for actin in establishment and


