ESCRT regulates surface expression of the Kir2.1 potassium channel

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ABSTRACT Protein quality control (PQC) is required to ensure cellular health. PQC is recognized for targeting the destruction of defective polypeptides, whereas regulated protein degradation mechanisms modulate the concentration of specific proteins in concert with physiological demands. For example, ion channel levels are physiologically regulated within tight limits, but a system-wide approach to define which degradative systems are involved is lacking. We focus on the Kir2.1 potassium channel because altered Kir2.1 levels lead to human disease and Kir2.1 restores growth on low-potassium medium in yeast mutated for endogenous potassium channels. Using this system, first we find that Kir2.1 is targeted for endoplasmic reticulum–associated degradation (ERAD). Next a synthetic gene array identifies nonessential genes that negatively regulate Kir2.1. The most prominent gene family that emerges from this effort encodes members of endosomal sorting complex required for transport (ESCRT). ERAD and ESCRT also mediate Kir2.1 degradation in human cells, with ESCRT playing a more prominent role. Thus multiple proteolytic pathways control Kir2.1 levels at the plasma membrane.

INTRODUCTION

Protein quality control (PQC) was initially described to reflect the fact that secreted proteins with various defects were unable to exit the endoplasmic reticulum (ER). Specifically, defects in protein glycosylation, folding, or assembly or protein aggregation led to ER retention (Hurtley and Helenius, 1989). More recently, it has become appreciated that PQC operates throughout the eukaryotic secretory pathway to ensure that toxic or misfolded proteins do not accumulate (Arvan et al., 2002; Apaja et al., 2010). In turn, regulated protein degradative systems provide a means to fine tune wild-type protein levels and thus their activities. Therefore even folded and functional proteins may be destroyed if existing levels are toxic or do not match cellular needs. If PQC or the regulated protein degradation system is defective or if the PQC machinery fails to recognize an aberrant protein, one of many human diseases may result (Gestwicki and Garza, 2012; Guerriero and Brodsky, 2012).

As noted, secretory pathway PQC begins in the ER, and proteins that fail to pass this step can be destroyed by ER-associated degradation (ERAD). ERAD substrates are first recognized by molecular chaperones and chaperone-like lectins, which target them for ubiquitination (Aebi et al., 2010; Claessen et al., 2012; Hebert and Molinari, 2012). During ubiquitination, ERAD substrates are retrotranslocated to the cytoplasm and delivered to and degraded by the 26S proteasome (Mehnert et al., 2010; Smith et al., 2011). If the ERAD machinery is overwhelmed or if aberrant proteins accumulate, the unfolded protein response is induced, and in some cases misfolded species can escape and advance to the Golgi. In these circumstances, ERAD substrates are returned to the ER (Vashist et al., 2001; Haynes et al., 2002; Taxis et al., 2002), or in yeast a Golgi quality control system, which is absent in mammalian cells, recognizes and delivers substrates to the vacuole for degradation via the multivesicular body (MVB) pathway (Chang and Fink, 1995; Hong et al., 1996; Spear and Ng, 2003; Wang and Ng, 2010; Coughlan et al., 2004). PQC mechanisms also exist at the plasma membrane, and in

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Abbreviations used: CFTR, cystic fibrosis transmembrane conductance regulator; ERAD, endoplasmic reticulum–associated degradation; ESCRT, endosomal sorting complex required for transport; MVB, multivesicular body; PQC, protein quality control; SGA, synthetic gene array.
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this case selected substrates are endocytosed and targeted to the lysosome (Huotari and Helenius, 2011). For example, the disease-associated \( \Delta F508 \) mutant form of the cystic fibrosis transmembrane conductance regulator (CFTR) is an ERAD substrate but is also less stable than wild-type CFTR when delivered to the plasma membrane by low-temperature correction. Genome- and protein-wide screens revealed a significant number of factors required for the disposal of \( \Delta F508 \) CFTR from the plasma membrane (Trzcinska-Danelut\'i et al., 2009; Okiyona et al., 2010), and for many substrates quality control at the plasma membrane also follows the MVBI pathway (Jenness et al., 1997; Arvan et al., 2002; Apaja et al., 2010; Keener and Babst, 2013; Zhao et al., 2013). There are multiple mechanisms to ensure that unwanted secreted proteins are destroyed, but it is generally unclear how these systems cooperate and which pathway might be most important for the PQC of a given substrate.

To begin to investigate these questions, we reengineered and optimized a yeast expression system for the Kir2.1 potassium channel because relatively small changes in the amount of active, plasma membrane–resident Kir2.1 can be detected (Tang et al., 1995; Nakamura and Gaber, 1998; Minor et al., 1999). Like other inward-rectifying potassium channels, Kir2.1 is formed by a tetrameric assembly of identical subunits; each subunit has a potassium selectivity sequence, which is flanked by two transmembrane domains. The N- and C- termini form a large regulatory structure that surrounds the central pore (Schwalbe et al., 2002). Tetrameric Kir2 potassium channels function primarily in heart, skeletal muscle, and neurons and consist of five family members—Kir2.1–2.4 and 2.6 (de Boer et al., 2010; Hibino et al., 2010). Kir2.1 is responsible for controlling cell excitability in many cell types, especially in heart and skeletal muscle, and loss-of-function mutations in Kir2.1 result in Anderson–Tawil syndrome (ATS-1), a disorder characterized by periodic paralysis, long QT ventricular arrhythmias, and skeletal muscular dysplasia (Plaster et al., 2001). Gain-of-function mutations in Kir2.1 lead to short QT syndrome, which can also cause cardiac arrhythmias (Priori et al., 2005). These cases illustrate the importance of maintaining proper Kir2.1 levels. However, the factors necessary to support Kir2.1 trafficking and stability have not been explored in significant detail—hence our desire to identify these components using a systems-level approach.

We discovered that Kir2.1 in yeast traffics to the plasma membrane but resides predominately in the ER, where most of the protein is targeted for ERAD. Maximal Kir2.1 degradation required the AAA-ATPase Cdc48, the ER-associated E3 ubiquitin ligases Hrd1 and Doa10, and the cytoplasmic Hsp70 chaperone Ssa1. Because Kir2.1 supports the growth of \( \Delta \text{trk}2\Delta \) yeast on low-potassium medium (Tang et al., 1995; Nakamura and Gaber, 1998), this system also provided a unique opportunity to identify the spectrum of PQC and trafficking factors necessary to maintain active Kir2.1 at the plasma membrane. Of interest, ERAD-requiring factors were absent from the list of yeast mutants that significantly improved growth on low potassium, suggesting that the level of functional Kir2.1 is not regulated by ERAD. In contrast, most endosomal sorting complex required for transport (ESCRT) components were isolated. These data indicate that the plasma membrane and Golgi quality control systems regulate Kir2.1 residence at the plasma membrane. Consistent with this hypothesis, ESCRT also regulated Kir2.1 residence at the plasma membrane in human cultured cells. Together our results provide the first genomic analysis of the myriad degradative systems that operate on a single protein.

**RESULTS**

**Kir2.1 is an ERAD substrate**

The Kir2.1 potassium channel most likely encounters multiple PQC checkpoints as it travels through the secretory pathway to the plasma membrane. For example, an ATS-1 disease–causing mutation in the channel, \( \Delta 314-315 \), prevents channel trafficking from the Golgi apparatus to the plasma membrane by virtue of its inability to associate with the AP-1 clathrin–associated adaptor (Ma et al., 2011). To gain a more comprehensive understanding of the Kir2.1 biosynthetic trafficking and degradation pathways, we refined a yeast expression system. To this end, a doubly tagged (myc and hemagglutinin [HA]) version of Kir2.1 was expressed under the control of a constitutive promoter. Analysis of Kir2.1 localization by indirect immunofluorescence microscopy primarily showed a perinuclear staining that colocalized with the ER-resident chaperone Kar2 (BiP; Figure 1A). Sucrose gradient centrifugation analysis indicated that a significant amount of Kir2.1 comigrated with Kar2 (Figure 1B, lanes 1–6). However, a fraction of Kir2.1 also comigrated with the plasma membrane protein Pma1 (lanes 9–11). These data suggest that whereas the majority of Kir2.1 is ER localized, a small pool advances to the plasma membrane. Thus the yeast expression system provides a model to explore PQC steps encountered by Kir2.1 as it travels through the secretory pathway.

Next the involvement of ERAD was assessed. For these studies, Kir2.1 stability was analyzed by cycloheximide chase in wild-type and mutant strains. We found that the channel was rapidly degraded, becoming almost undetectable within 90 min (Figure 2A). In \( \Delta \text{pdr5} \) yeast, which allows studies with proteasome inhibitors, MG132 partially stabilized Kir2.1 from degradation (Figure 2A). Further, the AAA-ATPase, Cdc48, which extracts ERAD substrates from the ER membrane–resident Kir2.1 can be detected (Tang et al., 1995; Nakamura and Gaber, 1998; Minor et al., 1999). Like other inward-rectifying potassium channels, Kir2.1 is formed by a tetrameric assembly of identical subunits; each subunit has a potassium selectivity sequence, which is flanked by two transmembrane domains. The N- and C- termini form a large regulatory structure that surrounds the central pore (Schwalbe et al., 2002). Tetrameric Kir2 potassium channels function primarily in heart, skeletal muscle, and neurons and consist of five family members—Kir2.1–2.4 and 2.6 (de Boer et al., 2010; Hibino et al., 2010). Kir2.1 is responsible for controlling cell excitability in many cell types, especially in heart and skeletal muscle, and loss-of-function mutations in Kir2.1 result in Anderson–Tawil syndrome (ATS-1), a disorder characterized by periodic paralysis, long QT ventricular arrhythmias, and skeletal muscular dysplasia (Plaster et al., 2001). Gain-of-function mutations in Kir2.1 lead to short QT syndrome, which can also cause cardiac arrhythmias (Priori et al., 2005). These cases illustrate the importance of maintaining proper Kir2.1 levels. However, the factors necessary to support Kir2.1 trafficking and stability have not been explored in significant detail—hence our desire to identify these components using a systems-level approach.

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**FIGURE 1:** Kir2.1 resides primarily in the yeast ER. (A) Indirect immunofluorescence microscopy of yeast expressing Kir2.1. Fixed cells were probed with antibodies against the ER marker Kar2/BiP and the HA tag to visualize Kir2.1. Right, merge showing extensive colocalization. (B) Lysates from cells expressing Kir2.1 were analyzed by centrifugation in a 30–70% sucrose gradient under conditions to maximize ER and plasma membrane isolation (Roberg et al., 1997). The gradient was fractionated from the top (fraction 1) to the bottom of the tubes (fraction 13). The migration of Kir2.1, the ER chaperone, Kar2/BiP, and the plasma membrane protein Pma1 were evaluated by Western blot analysis.
the ER membrane and into the cytosol (Wolf and Stolz, 2012), was required for degradation, since Kir2.1 was completely stabilized in a temperature-sensitive mutant, cdc48-2, at a nonpermissive temperature (Figure 2B). Combined with the data in Figure 1, these results establish Kir2.1 as an ERAD substrate in yeast.

To determine whether Hsp70 chaperones facilitate the ERAD of Kir2.1, we expressed the protein in the temperature-sensitive Ssa1 mutant ssa1-45. The cytosolic Hsp70, Ssa1, is required for the degradation of ERAD substrates with misfolded cytosolic domains (Hill and Cooper, 2000; Zhang et al., 2001; Han et al., 2007; Nakatsukasa et al., 2008), and in line with these data, Kir2.1 degradation was attenuated at the nonpermissive temperature in the ssa1-45 strain (Figure 2C). In contrast, Kir2.1 degradation was unaffected in a kar2-1 mutant, which is required for the ERAD of substrates with misfolded lumenal domains (Plemper et al., 1997; Brodsky et al., 1999; Hill and Cooper, 2000; Figure 2D). These observations suggest that Kir2.1 folds poorly in yeast and is recognized as an ERAD substrate by the cytosolic Hsp70, Ssa1. As described later, Kir2.1 is also targeted for ERAD in mammalian cells.

Nearly all ERAD substrates in yeast are ubiquitinated by the E3 ligases Hrd1 and/or Doa10 (Claessen et al., 2012). To investigate whether Kir2.1 was degraded in an Hrd1- and/or Doa10-dependent manner, we expressed Kir2.1 in hrd1Δ, doa10Δ, hrd1Δdoa10Δ, and HRD1DOA10 strains. We found that Kir2.1 was stabilized to a similar level when either HRD1 or DOA10 was deleted, and when the genes encoding both E3s were absent, the protein was further stabilized (Figure 3A). We next asked whether deletion of the E3 ubiquitin ligases led to a decrease in Kir2.1 ubiquitination. Kir2.1 was immunoprecipitated from wild-type or hrd1Δdoa10Δ yeast and ubiquitin levels were examined by immunoblotting. As anticipated, deleting HRD1 and DOA10 led to ~40% reduction in Kir2.1 ubiquitination (Figure 3, B and C). Also as anticipated, an analysis of Kir2.1 residence by density centrifugation (see earlier discussion) in the hrd1Δdoa10Δ strain established that the protein accumulated primarily in the ER and to some extent in the Golgi; however, there was no significant increase in the plasma membrane pool, as defined by comigration with Pma1 (Supplemental Figure S1). Because Kir2.1 degradation is Ssa1 dependent (see earlier discussion), we anticipated the requirement for Doa10, which adds ubiquitin onto ERAD substrates with misfolded cytoplasmic domains, that is, ERAD-C substrates (Vashist and Ng, 2004; Carvalho et al., 2006; Denic et al., 2006). The more surprising Hrd1 dependence suggested that Kir2.1 folding defects are also recognized within the membrane (Sato et al., 2009). Thus the Kir2.1 folding lesions in yeast are complex.

A yeast genomic screen identifies regulators of Kir2.1 biogenesis

To identify the spectrum of factors that affect Kir2.1 PQC, we performed a genomic screen. For these studies, we took advantage of the ability of Kir2.1 to rescue the growth of a yeast strain lacking the Trk1 and Trk2 potassium transporters (Tang et al., 1995; Nakamura and Gaber, 1998). A synthetic gene array (SGA) query strain was engineered in which TRK1 and TRK2 potassium transporters (Tang et al., 1995; Nakamura and Gaber, 1998) were deleted and Kir2.1 was expressed. In low-potassium medium, growth of the strain correlated with Kir2.1 activity (Tang et al., 1995; Nakamura and Gaber, 1998). Thus PQC factors can be identified in an SGA screen as mutants that enhance cell growth in a Kir2.1-dependent manner. We ultimately determined that expressing Kir2.1 from a TEF promoter on a centromeric plasmid (Supplemental Figure S2A) and then
were screened on selective medium at pH 4 in the absence of added potassium when Kir2.1 was expressed (Supplemental Figure S3). A representative Western blot from one experiment is shown below. (B) Kir2.1 expressed in either wild-type (lane 1) or hrd1Δdoa10Δ (lane 2) mutant yeast strains was immunoprecipitated from lysates with anti-HA-agarose beads, and Kir2.1 and conjugated ubiquitin were analyzed by Western blot analysis. The result from immunoprecipitation using cells containing a vector control is shown for comparison (lane 3). (C) Relative ubiquitination of Kir2.1 immunoprecipitated from wild-type and hrd1Δdoa10Δ yeast. The graph represents means ± SE from 13 precipitated samples of each strain, each from a distinct clone.

Propagation on medium lacking potassium at pH 4 (Nakamura and Gaber, 1998) and 30°C (Supplemental Figure S2B) provided the optimal set of conditions to screen for genes that, when mutated, would augment growth on low potassium. As a control, we found that the residence of Kir2.1, as measured in sucrose gradient analyses as in Figure 1, was unaffected when cells were propagated at pH 4 (unpublished data).

Preventing the ERAD of some proteins increases their folding and secretion efficiencies (Qu et al., 1996; Halaban et al., 1997; Mitchell et al., 1998; Schubert et al., 1998; Vij et al., 2006; Kincaid and Cooper, 2007; Grove et al., 2009; Fisher et al., 2011). Consistent with Kir2.1 perhaps behaving in a similar manner, deletion of DOA10 subtly increased growth of the trk1Δtrk2Δ strain on low potassium when Kir2.1 was expressed (Supplemental Figure S3).

Having established that the system may detect PQC factors for Kir2.1, we conducted a genomic screen. First, the query strain was crossed to the yeast deletion collection using established SGA methodology (Tong and Boone, 2006). Next the resulting haploid strains, which each lacked one of 4848 nonessential yeast genes, were screened on selective medium at pH 4 in the absence of added potassium ([K+] < 10 mM; Nakamura and Gaber, 1998). A total of 271 strains that exhibited significantly increased growth on low potassium were identified (see Figure 4A for one example). To focus our efforts, we eliminated 36 strains with mutated genes that compromised select biosynthetic pathways or represented characterized transcription and translation factors. In addition, 20 strains containing dubious open reading frames were not considered further.

Fresh isolates of the remaining 215 strains were crossed to the query strain containing either a vector control or the Kir2.1 expression vector and rescreened under a higher level of stringency to eliminate possible false positives. Added into this analysis were strains lacking genes that encode members of protein complexes (e.g., ESCRT; see later discussion) that featured prominently in the preliminary screen. The 95 strongest hits from this second-round screen were selected and ranked by measuring growth in liquid culture in 96-well plates. The ratio of the doubling time of a vector-only control strain to the same strain expressing Kir2.1 was calculated to produce a growth score. The analysis identified 54 deletion strains that reproducibly increased growth in a Kir2.1-dependent manner, each having a significantly higher growth score than the query strain containing the vector-only control (Figure 4B, Table 1, and Supplemental Table S1). To our surprise, genes required for ERAD were completely absent from this list, suggesting that the modest increase in growth when DOA10 was deleted (Supplemental Figure S2) was below our cutoff. Instead, nearly all of the identified genes are associated with secretory pathway function at or beyond the Golgi. For example, a member of the phosphatidylinositol 3-kinase complex (Vps38) that regulates carboxypeptidase Y (CPY) sorting and interacts with the retromer complex represented the strongest hit (Kihara et al., 2001; Burda et al., 2002). Also prominent among the hits were a large number of ESCRT proteins (Did2, Vps36, Vps27, Vps22, Vps2, Vps23, Vps37, Mvb12, and Vta1).

ESCRT is involved in Golgi and plasma membrane quality control (Li et al., 1999; Reggiori and Pelham, 2002; Apaja et al., 2010; Okiyonda et al., 2010; Wang et al., 2011), so we directly examined the role of select ESCRT members in Kir2.1 protein biogenesis. First, a VPS23-null strain (vps23Δ) strain, which also lacked TRK1 and TRK2, was constructed, and after introduction of the Kir2.1 expression vector, the yeast were rescreened on low-potassium medium. Improved growth by Kir2.1 in the trk1Δtrk2Δvps23Δ strain was quite prominent but was significantly decreased in a trk1Δtrk2ΔVPS23 background (Figure 5A). To confirm that rescue on low-potassium medium was associated with Kir2.1 activity, we created a well-characterized loss-of-function mutation in Kir2.1, replacing the potassium selectivity filter 144GYG146 sequence with 144AAA146 (Tinker et al., 1996). After the expression plasmid was introduced into cells, the inactive channel was unable to rescue growth, even though it...
The results presented in the preceding sections indicate that Kir2.1 is an ERAD substrate in yeast but that later steps in the secretory pathway play a more profound role in controlling the population of active Kir2.1 at the plasma membrane. To examine whether these phenomena were evident in mammalian cells, we examined Kir2.1 stability in HeLa cells.

Cycloheximide chase analysis confirmed that Kir2.1 was degraded by the 26S proteasome, as it was in yeast, although to a lesser extent (Figure 6, A and B). Because the majority of wild-type Kir2.1 turnover was proteasome independent, we next examined whether Kir2.1 was degraded in the lysosome. Consistent with a role for lysosomal degradation in controlling Kir2.1 levels, leupeptin increased Kir2.1 steady-state levels, as reported (Jansen et al., 2008; Nalos et al., 2011; Figure 6, C and D). Further, leupeptin addition augmented colocalization with a late endosomal/lysosomal marker, CD63 (Figure 6E). In metabolic pulse-chase experiments, treatment with leupeptin again stabilized Kir2.1 in HeLa cells (Supplemental Figure S6) and, as anticipated, MG132 addition led to more modest effects (unpublished data).

To determine whether ESCRT was required for the lysosome-dependent degradation of Kir2.1, we depleted the ESCRT components Tsg101 (an ESCRT-I component that is the yeast Vps23 homologue; Rothman and Stevens, 1986; Robinson et al., 1988; Babst et al., 2000) and HRS (an ESCRT-0 component that is a homologue of yeast Vps36) from HeLa cells (Supplemental Figure S4). Proteasome- and ESCRT-dependent degradation of Kir2.1

In human cells

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We demonstrated that Kir2.1 is subject to PQC at multiple levels in both yeast and human cells. An initial characterization of Kir2.1 in yeast revealed a majority of the channel residing in the ER, of yeast Vps27; Robinson et al., 1988; Komada et al., 1997; Bilodeau et al., 2002). Treatments with targeted small interfering RNAs (siRNAs) led to nearly complete absence of Tsg101 and ~50% decrease in the amount of HRS (Figure 6, F and G). In parallel, there was a corresponding increase in Kir2.1 abundance (Figure 6H). Addition of leupeptin did not lead to a further increase in Kir2.1 when the ESCRT components were knocked down, consistent with delivery to and degradation of the channel in the lysosome (Figure 6I). Our collective data from the yeast and human cell models demonstrate that ESCRT-dependent targeting of Kir2.1 to the vacuole/lysosome regulates the levels of Kir2.1 at the plasma membrane. These results are also the first to implicate ESCRT in the regulation of a Kir potassium channel.

**DISCUSSION**

We demonstrated that Kir2.1 is subject to PQC at multiple levels in both yeast and human cells. An initial characterization of Kir2.1 in yeast revealed a majority of the channel residing in the ER,
where it is targeted for ERAD. In contrast, our genomic screen revealed that the abundance of active Kir2.1 at the plasma membrane is controlled by vacuolar targeting factors. These data suggest that a larger, permanently misfolded population of Kir2.1 is delivered to the ERAD pathway, which may be unable to mature or become active. By contrast, the pool of Kir2.1 that migrates beyond the ER appears to be in a folding-competent state, in which it is regulated by ESCRT and associated factors (see later discussion). In one model, this population may transiently adopt misfolded conformations, and defects in ESCRT-mediated degradation could then rescue this protein pool so it functions at the plasma membrane. The notion of an unstable population of maturing and even functional ion transporters is not new (Hill and Cooper, 2000; Helliwell et al., 2001; Lu et al., 2007; Apaja et al., 2010), but our data represent the first hint of the relative strengths of unique quality control mechanisms that may act on a single protein in the secretory pathway.

To some extent the studies in HeLa cells validated the use of yeast as a model to identify the pathways that affect Kir2.1 biogenesis. Kir2.1 is regulated by both ERAD and ESCRT-mediated lysosomal degradation, as in yeast, but the relative contribution of each is inverted. In yeast, the majority of Kir2.1 was degraded by ERAD, with a minor Kir2.1 population being targeted to the vacuole. Based on our calculations, rescue of trk1Δtrk2Δ yeast growth requires very few active Kir2.1 channels on the surface, so the screen provides a sensitive method to detect this minor pool. Nevertheless, rapid ERAD-mediated turnover precluded our ability to reproducibly measure changes in Kir2.1 organelle residence in the mutant strains (unpublished data). In contrast, Kir2.1 is primarily degraded in the lysosome in HeLa cells, whereas a smaller population of Kir2.1 is targeted for ERAD, and based on this fact, significant changes in the intraorganellar pool were evident (Figure 6).

One explanation for the relative instability of Kir2.1 in yeast may be unique lipid compositions. Lipids such as cholesterol and phosphatidylinositol 4,5-bisphosphate (PI-4,5-bisP) alter Kir2.1 function and may influence its stability (Huang et al., 1998; Romanenko et al., 2004). Yeast lack cholesterol (but contain ergosterol), and the levels of PI-4,5-bisP might differ significantly between yeast and human cells. In addition, yeast could lack a Kir2.1 cofactor, which stabilizes the protein. For example, a yeast screen was used to identify cofactors for a rodent monocarboxylate transporter that increased transporter levels and function (Makuc et al., 2004). It is unknown whether a Kir2.1 cofactor exists in mammals, but a yeast-based screen provides a route to identify this putative factor, as well as intragenic mutations that affect Kir2.1 function and PQC.

Numerous ESCRT components were identified in the screen (Table 1 and Supplemental Table S1). Substrate selection in the MVB pathway begins with the recognition and clustering of ubiquitinated cargo by ESCRT-0 (e.g., Vps27, which was identified in the screen; Bilodeau et al., 2002; Wollert and Hurley, 2010; Mayers et al., 2011). ESCRT-I (Vps23, Vps37, and Mvb12 were represented in the screen) and ESCRT-II (Vps22 and Vps36 were isolated) are then recruited and confine cargo at the site of vesicle formation, which initiates vesicle invagination before ESCRT-III (e.g., Vps2, Table 1) is recruited (Teo et al., 2004, 2006; Gill et al., 2007; Kostelansky et al., 2008).

We also identified other factors that regulate Golgi trafficking and vacuolar function (Table 1). Deletion of VPS38, which encodes a component of phosphatidylinositol 3-kinase complex II (PI3KII), most strongly affected Kir2.1-dependent rescue. PI3K is a member of two complexes, PI3K and PI3KII. PI3K localizes primarily to the vacuole and is involved in autophagy, whereas PI3KII resides in the Golgi and endosome and regulates protein trafficking from the endosome to the Golgi (Burdà et al., 2002; Obara et al., 2006). Deletion of the genes encoding Vps38 and another component of the PI3K complexes, Vps30, compromises retromer activity (Burdà et al., 2002). Retromer functions in the recovery of proteins from the endosome to the Golgi (Seaman et al., 1998), and all five members of the retromer complex (Vps5, Vps17, Vps26, Vps29, and Vps35) are represented within the 20 strongest hits in the screen. Although the mechanism is undefined, deletion of retromer increases plasma membrane residence of other membrane proteins in yeast, flies, and worms (Kim et al., 2007; Korolchuk et al., 2007; Dang et al., 2011). More recently, the receptor activator of NF-κB (RANK), which is involved in osteoclast formation, was shown to be inhibited by retromer-mediated transport from the endosome to the Golgi, thereby increasing RANK at the plasma membrane in murine osteoclasts (Xia et al., 2013). Inhibition of retromer may cause a factor that would normally antagonize Kir2.1 plasma membrane residence, such as a Golgi retention factor, to be degraded, or retromer may aid in retrieving a population of Kir2.1 that traffics through an endosomal intermediate en route to the plasma membrane.

Additional proteins that regulate the trafficking/localization of proteins at the Golgi and endosome were identified as weaker hits. These include Golgi-associated GTPases (Arl1, Arl3, Ypt6) that regulate Golgi–endosome trafficking (Li and Warner, 1996; Luo and Chang, 1997; Behnia et al., 2004). Ypt6 interacts with Vps52 in the Golgi-associated retrograde protein (GARP) complex, which retrieves proteins from the endosome to the Golgi; two GARP components were among the hits (Vps52 and Vps53; Conibear and Stevens, 2000; Siniossoglou and Pelham, 2002). Other protein
FIGURE 6: Kir2.1 degradation is primarily lysosomal dependent and requires ESCRT. (A, B) Kir2.1 stability was analyzed by a cycloheximide chase in HeLa cells. HeLa cells transfected with pcDNA3.1-HA-Kir2.1 were treated with 15 μM MG132 or dimethylsulfoxide and harvested, lysed, and analyzed by SDS–PAGE. Kir2.1 was detected with an anti-HA antibody. Actin levels served as a loading control. (A) A representative blot. (B) Data represent means of four or five experiments ± SD. (C) HeLa cells transfected with HA-Kir2.1 were treated with 100 μM leupeptin for 18 h. Cells were then lysed and visualized by Western blot. (D) Means ± SE from three independent trials from part C treated with or without leupeptin. (E) Confocal microscopy was performed in HeLa cells transfected with pcDNA3.1-HA-Kir2.1. Cells were treated with 100 μM leupeptin as described in Materials and Methods and were mounted in medium containing DAPI. Merge, colocalization of Kir2.1 (green) and CD63 (red), a marker of endosomes/lysosomes (yellow). (F–I) HeLa cells were cotransfected with the indicated siRNA and pcDNA3.1-HA-Kir2.1, and after 72 h, cells were harvested and the levels of Kir2.1, TSG101, HRS, and tubulin measured. Where indicated, 100 μM leupeptin was added to cells for 18 h. Tubulin served as a loading control. (H) The levels of Kir2.1 when TSG101 or HRS were knocked down (lane 3 from F and G) were normalized to the scramble control (lane 1 from F and G) to determine the effect of siRNA on Kir2.1 abundance. The data represent means ± SE of six to eight independent trials. (I) Levels of Kir2.1 in the presence of leupeptin when TSG101, HRS, or scramble siRNAs were transfected and normalized to Kir2.1 levels when leupeptin was absent (lanes 1 and 3; open bars) to determine the magnitude of the leupeptin response. The data represent means ± SE of six to eight independent trials. *p < 0.05, **p < 0.01, ***p < 0.001.
complexes involved in trafficking within the Golgi, such as the TRAPP complex (Tca17, Tns85; Sacher et al., 2000; Montpetit and Conibear, 2009) and the conserved oligomeric Golgi (COG) complex (Cog5, Cog6, Cog7, Cog8; Ram et al., 2002; Ungar et al., 2002), were among the 18 weakest hits, but whether these play a direct or indirect role in Kir2.1 trafficking is unclear. Nevertheless, these data are consistent with Golgi accumulation of the Kir2.1 Andersen–Tawil mutant (Ma et al., 2011).

Vacuole targeting of proteins via ESCRT requires ubiquitination by Rsp5 or Ttt1 (Helliwell et al., 2001; Hicke and Dunn, 2003; Dupre et al., 2004; Katzmann et al., 2004; Pizzirusso and Chang, 2004; Wang et al., 2011). Rsp5 is an essential protein, so it would have been absent from our screen, but the Rsp5 adaptors proteins Bsd2 and Tre1, which regulate the trafficking and stability of plasma membrane and Golgi proteins, were identified (Portnoy et al., 2000; Hettema et al., 2004; Stimpson et al., 2006). Consistent with these results, the plasma membrane–resident population of Kir2.1 is most likely regulated by ubiquitin conjugation (Alesutan et al., 2011).

The rescue of a trk1Δtrk2Δ strain on low potassium when each of the identified genes was deleted was mediated by Kir2.1 function and is not simply a result of the deleted gene making the yeast more tolerant to potassium-poor medium. For each hit, we showed that the introduction of a vector control was unable to improve growth to the same level as a strain expressing Kir2.1. However, a screen for mutants that exhibited increased sensitivity to cationic drugs identified Ari1, the COG complex, the GARP complex, and retromer (Barreto et al., 2011), suggesting that some of hits from our screen may alter the localization of other yeast potassium channels in addition to Kir2.1. Many of our hits also lead to increased CPY secretion (Bonangelino et al., 2002). However, the strains that increase CPY secretion do not completely overlap with the hits identified in this study; thus Kir2.1 localization is regulated differently than this soluble, vacuolar-resident protein (see Supplemental Table S1). In fact, there is more overlap with genes identified in a screen for mutants that rescue the localization of a defective form of the plasma membrane ATPase Pma1 (e.g., Vps38, Vps36, Vps29, Vps13, Vps35, Vps27, and Bsd2; Luo and Chang, 1997). A screen was also performed in which another inward-rectifying potassium channel, Kir3.2, was mutated so that it was sodium selective, and strains expressing the protein were crossed to a subset of the deletion collection (Haass et al., 2007). The resulting haploids were screened on medium containing high sodium, and seven genes associated with COPII-dependent transport and lipid biogenesis were identified that, when mutated, reduced sodium-mediated toxicity.

Our data contribute to a growing number of substrates that are regulated by the PQC machinery at the Golgi and plasma membrane in yeast (Chang and Fink, 1995; Hong et al., 1996; Jenness et al., 1997; Haynes et al., 2002; Spear and Ng, 2003; Coughlan et al., 2004; Wang and Ng, 2010) and mammals (Armstrong et al., 1999; Ashok and Hegde, 2009; Apaja et al., 2010; Okiyoneda et al., 2010). It is worthwhile to note that the population of an unrelated potassium channel, KCa3.1, at the plasma membrane is also regulated by ESCRT and that mutant forms of this channel are subject to ERAD (Balut et al., 2012). Therefore in the future it will be interesting to determine whether ESCRT is used for the targeted destruction of all or only a subset of potassium and perhaps other ion channels. In addition, we found that a core collection of Hsp70 and Hsp40 molecular chaperones aid in the identification of Kir2.1 as an ERAD substrate in the ER. Studies in mammalian cells identified Hsc70, Hsp90, and Hsp40s as important for the recognition of the CFTR mutant that resides at the plasma membrane (Okiyoneda et al., 2010). Future work will uncover whether these and other chaperones operate at multiple quality control networks for Kir2.1 and other ion transporters.

**MATERIALS AND METHODS**

**Yeast strains and growth conditions**

Yeast strains were propagated at 26°C, and standard methods for growth, media preparation, and transformation were used unless indicated otherwise (Adams et al., 1998). Yeast with plasmids containing constitutive promoters (GPD or TEF) were grown in selective medium and harvested at the OD_{600} indicated in each experiment. Yeast harboring plasmids containing the MET25 promoter were maintained on medium containing 0.5 mM methionine to inhibit protein synthesis. To induce Kir2.1 expression, cells were grown to log phase in selective medium supplemented with methionine, collected by centrifugation and washed in sterile water, and then resuspended in selective medium lacking methionine for 1–2 h. After induction, cells were used as described for each experiment. For low-potassium growth assays and the SGA screen, 20 mM MES (2-[(N-morpholino)ethanesulfonic acid] and the indicated concentration of potassium were added. Medium was also prepared at the desired pH with Tris-HCl. A complete list of the strains used in this study is presented in Supplemental Table S2.

For growth assays, 5-ml cultures were grown to log phase in synthetic complete medium lacking leucine (SC-Leu) but containing 100 mM KCl at 30°C, and OD_{600} of cells was collected by centrifugation. The yeast were resuspended in 500 µl of sterile water. Cells were serially diluted 10-fold, plated using a 32-pin manifold onto SC-Leu medium containing 0, 10, 25, or 100 mM KCl, and grown at 30°C for 2 d. Plates were imaged using an Epson Perfection 3490 Photo Scanner. To assay protein expression, an equal number of cells was taken from the plates, and total protein was precipitated with 10% trichloroacetic acid and resolved by SDS–PAGE before Western blot analysis (Zhang et al., 2001). Kir2.1 was detected using an anti–HA-horseradish peroxidase (HRP)–conjugated antibody, and blots were probed with anti–glucose-6-phosphate dehydrogenase (G6PD) antiserum to provide a loading control. The G6PD primary antibody was decorated with donkey HRP-conjugated anti–rabbit immunoglobulin G secondary antibody. The Supersignal Chemiluminescent Substrate (Pierce, Rockford, IL) was used to develop the blots, and the signals were quantified using a Kodak 440CF Image Station and the associated Kodak 1D software (Eastman Kodak, Rochester, NY).

Strain YAK01 (Supplemental Table S2) was constructed from Y7029 by homologous recombination (Brachmann et al., 1998). The NAT cassette was PCR amplified using primers oAK01 and oAK02 (Supplemental Table S3), which also contained homology to the TRK2 locus, and the PCR product was used to delete TRK2 in Y7029. The URA3 cassette from pRS426 (Mumberg et al., 1995) was PCR amplified using primers oAK05 and oAK06 (Supplemental Table S3), which contained homology to the TRK1 locus. The product was then used to delete TRK1 in Y7029. YAK02 and YAK03 were constructed from AK01 by mating this strain with a strain containing constitutive promoters (GPD or TEF) on a plasmid. All constructed strains were confirmed by growth on selective medium and harvested at the OD_{600} indicated in each experiment. Yeast harboring plasmids containing the MET25 promoter were maintained on medium containing 0.5 mM methionine to inhibit protein synthesis. To induce Kir2.1 expression, cells were grown to log phase in selective medium supplemented with methionine, collected by centrifugation and washed in sterile water, and then resuspended in selective medium lacking methionine for 1–2 h. After induction, cells were used as described for each experiment. For low-potassium growth assays and the SGA screen, 20 mM MES (2-[(N-morpholino)ethanesulfonic acid] and the indicated concentration of potassium were added. Medium was also prepared at the desired pH with Tris-HCl. A complete list of the strains used in this study is presented in Supplemental Table S2.

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Plasmid construction

Mouse Kir2.1 in pcDNA3.1 was modified with an internal HA tag that was positioned so that it faced the extracellular space (Ma et al., 2001). To create a yeast Kir2.1-HA expression construct with a C-terminal myc tag (myc-Kir2.1-HA), the Kir2.1 coding sequence within the pcDNA3.1 vector was PCR amplified using the PCR Master Mix reagent (Fermentas, Waltham, MA). The amplified Kir2.1 sequence was purified using the PureLink PCR purification kit (Invitrogen) and ligated into the pGEM-T easy vector (Promega, Madison, WI). The pGEM-T-Kir2.1 plasmids were sequenced to identify clones that contained the correct Kir2.1 insert. The insert was removed from pGEM-T using EcoRI, the fragment was gel purified using PureLink Quick Gel Extraction Kit (Invitrogen), and the isolated species was ligated into the EcoRI site of yeast plasmids containing either constitutive (TEF, GPD) or regulated (MET25) promoters (Mumberg et al., 1994, 1995). The myc-Kir2.1-HA insert was then cloned into digested pRS vectors containing different promoters and genes for auxotrophic selection (Mumberg et al., 1994, 1995) using SmaI and Xhol. The cut vector was treated with Antarctic phosphatase (New England BioLabs, Ipswich, MA), and the digested vectors were run on a 1% agarose gel and purified with PureLink Quick Gel Extraction Kit (Invitrogen). The cut vectors and inserts were finally ligated using T4 DNA Ligase (Fermentas). Kir2.1-AAA and Kir2.1A334-315 were made by two-stage PCR mutagenesis (Vallejo et al., 1994). The Kir2.1 mutated cassettes were then inserted into the PRS415TEF vectors as described. All isolated inserts were subject to DNA sequence analysis.

Genetic screening conditions

The query strain (YAK01) was mated to the MATa strain deletion collection (Invitrogen) as described (Tong and Boone, 2006). In brief, the query strain was plated onto SC-Leu plates using a 96-pin manifold (Aladin Enterprises, Brisbane, CA). Strains in the deletion collection were then plated over the query strain, and the plates were incubated at 30°C for 1 d. The resulting diploid cells were plated onto SC-Leu containing 200 mg/l G418 (Research Products International), 100 mg/l clonNAT (Werner BioAgents, Jena, Germany), and 100 mM KCl to select for diploids and to maintain a potassium-sensitive phenotype. The released proteins were subjected to SDS–PAGE before the start of the chase, and the incubation was continued at 37°C for 10 min before performing the chase at 37°C. The cdc48-2 temperature-sensitive strain and the isogenic wild type were incubated at 39°C for 2 h before the start of the chase, and the incubation was continued at 39°C. A 1-ml aliquot of the culture was collected at the indicated time points, and pelleted cells were washed with ice-cold water containing protease inhibitors, flash-frozen in liquid nitrogen, and stored at −80°C. Total protein was precipitated from samples as described (Zhang et al., 2001) and immediately resolved by SDS–PAGE before Western blot analysis, as described.

To measure the relative amount of Kir2.1 ubiquitination, cells harboring the pRS426MET25-Kir2.1 plasmid were grown to an OD600 of 1.0 in 50-ml cultures of selective medium containing 0.5 mM methionine. The yeast were collected by centrifugation, washed in sterile water, and resuspended in the same medium lacking methionine for 2 h. After induction, the cells were collected by centrifugation, washed, and SDS sample buffer was used to liberate the bound proteins. The released proteins were subjected to SDS–PAGE and Western blot analysis to detect total Kir2.1 and the ubiquitinated Kir2.1 fraction.

The intracellular residence of Kir2.1 was determined by sedimentation in a sucrose gradient essentially as described (Sullivan
et al., 2003). A 40-ml culture was grown to an OD600 of 0.8, and the cells were harvested by centrifugation, resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 10% sucrose, and then disrupted by agitation with glass beads. The cell lysates were cleared of debris by low-speed centrifugation and the resulting lysate (300 μl) was layered at the top of a 11 ml 30–70% sucrose gradient and centrifuged at 100,000 × g in a Beckman SW41 rotor for 14 h at 4°C. Fractions were collected by pipetting from the top of the tube. The presence of specific proteins was analyzed by SDS–PAGE and Western blot.

Analysis of Kir2.1 in HeLa Cells
HeLa cells were cultured in DMEM with 10% FBS, 100 U/ml penicillin/streptomycin, and 2 mM glutamine at 37°C and 5% CO2. XtremeGENE9 transfection reagent (Roche, Indianapolis, IN) was used as per the manufacturer’s guidelines to transfect mouse Kir2.1 in pcDNA3.1 (pcDNA3.1-HA-Kir2.1). To knock down the expression of ESCRT proteins, HeLa cells plated in six-well dishes were cotransfected with 1 μg of pcDNA3.1-HA-Kir2.1 and 2 μg of siRNA using XtremeGENE siRNA transfection reagent (Roche, Penzberg, Germany). The siRNAs for human TSG101 (CCAAUA-UUCCUCAACUGC), human HRS (HGS; GAACCCACACGUCGCUU), and a scramble control (ON-TARGETplus nontargeting siRNA #1) were obtained from Invitrogen. After 54 h a final concentration of 100 μM leupeptin was added where indicated. After another 18 h the cells were washed with PBS and collected in 1% Triton in PBS with Protease Inhibitor Cocktail (P8340; Sigma-Aldrich). Lysates were rotated for 1 h at 4°C, followed by centrifugation at 13,000 rpm in a microcentrifuge for 5 min at 4°C. Supernatants were incubated with loading buffer for 30 min at room temperature, and proteins were analyzed by SDS–PAGE and Western blot.

To determine the extent to which Kir2.1 accumulates in MVBs in HeLa cells, Kir2.1-HA was colocalized with the MVB marker, CD63 (Escola et al., 1998), using a rabbit monoclonal HA antibody and a mouse monoclonal CD63 antibody. For these studies, cells were grown on glass coverslips and then fixed (4% paraformaldehyde, 30 min, 5°C), permeabilized with 0.1% Triton X-100 (10 min, 5°C), and blocked in 5% FBS (30 min, 5°C). The cells were then incubated with the primary antibodies (1:100, 2 h, 5°C), washed, and incubated with anti-mouse and anti-rabbit Alexa Fluor–conjugated secondary antibodies (1:250) for 1 h at room temperature. After washing, the cells were mounted in VectaShield (Vector Laboratories, Burlingame, CA) mounting media containing the nuclear marker 4’,6-diamidino-2-phenylindole (DAPI). The signals corresponding to each protein were visualized with a Zeiss 510 confocal microscope using a 63x oil immersion lens (numerical aperture 1.4), and images were processed using Volocity image analysis software (PerkinElmer, Boston, MA).

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

To monitor Kir2.1 stability by cycloheximide chase analysis, a final concentration of 50 μg/ml cycloheximide (Sigma-Aldrich) in the presence or absence of 15 μg/ml MG132 (Peptides International, Louisville, KY) was added to cells expressing Kir2.1-HA to stop protein translation and inhibit the proteasome, respectively. The cells were incubated at 37°C and harvested with 100 μl of lysis buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 150 mM NaCl, cComplete Mini protease inhibitor cocktail [Roche Diagnostics]), and the protein concentration was measured by bicinchoninic acid (Thermo Scientific). Equal amounts of protein were resolved by SDS–PAGE before Western blot analysis.

To assess the turnover of Kir2.1 using a metabolic pulse-chase protocol, HeLa cells were transfected with pcDNA3.1 HA-Kir2.1 and treated with 100 μM leupeptin immediately after transfection and again 6 h later. The following day, fresh leupeptin was added to DMEM lacking cysteine and methionine (Sigma-Aldrich), and cells were starved for 45 min, followed by a 1-h pulse with 125 μCi/ml EasyTag Express 35S protein labeling mix (PerkinElmer). After the pulse, the cells were chilled to 4°C using an ice-cold metal plate and washed three times for 5 min each with DMEM containing 5 mM cysteine and methionine and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4. The cells were then incubated with prewarmed chase media supplemented with 0.2% dimethylsulfoxide or 100 μM leupeptin. Leupeptin was readded at 3 and 6 h of the chase. At each measured time point, the cells were cooled by washing with ice-cold PBS, followed by solubilization with 500 μl of detergent solution (50 mM Tris, pH 8, 100 mM EDTA, pH 8, 0.4% sodium deoxycholate, 1% Nonidet P-40, protease inhibitors [1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 0.5 μg/ml pepstatin A]). The resulting lysates were centrifuged for 10 min at 17,000 × g in a refrigerated microcentrifuge, and the postnuclear supernatants were transferred to a fresh tube containing 10 μg of mouse anti-HA (12CA5; Roche) and rotated overnight at 4°C. Antibody-Kir2.1 complexes were captured using fixed Staphylococcus aureus (Pansorbin; Calbiochem, San Diego, CA) and washed three times with 10 mM Tris-HCl, 0.15 M NaCl, 1% Triton X-100, 1% Nonidet P-40, and 0.1% SDS, pH 7.4. Samples were eluted with SDS sample buffer supplemented with 5% β-mercaptoethanol and electrophoresed using 10% SDS–PAGE. The gels were dried and exposed to a phosphorimager screen before analysis using Image J.

Statistical analysis
To analyze experiments performed in HeLa cells (Figure 6), statistical analysis was performed using Prism (GraphPad, La Jolla, CA). Statistical significance was determined by one-way analysis of variance (ANOVA) and Tukey’s post hoc test in G and two-way ANOVA and Bonferroni post tests in H.

Antibodies
The following primary antibodies were used for studies in yeast: HRP-conjugated rat monoclonal anti-HA high-affinity (3F10; Roche), anti-HA mouse monoclonal (Roche), G6PD rabbit polyclonal antibody (Sigma-Aldrich), a ubiquitin (P4D1) mouse monoclonal antibody from Santa Cruz (Dallas, TX; sc-8017), and a polyclonal rabbit anti-Kar2 (Brodsky and Schekman, 1993). A polyclonal rabbit anti-Pma1 was a gift from Amy Chang (University of Michigan, Ann Arbor, MI). The following secondary antibodies were used in yeast: HRP-conjugated goat anti-mouse and goat anti-rabbit antibodies (Jackson ImmunoResearch, West Grove, PA) and Alexa Fluor 568 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse (Invitrogen, Penzberg, Germany). The siRNAs for human TSG101 (CCAAUA-UUCCUCAACUGC), human HRS (HGS; GAACCCACACGUCGCUU), and a scramble control (ON-TARGETplus nontargeting siRNA #1) were obtained from Invitrogen. After 54 h a final concentration of 100 μM leupeptin was added where indicated. After another 18 h the cells were washed with PBS and collected in 1% Triton in PBS with Protease Inhibitor Cocktail (P8340; Sigma-Aldrich). Lysates were rotated for 1 h at 4°C, followed by centrifugation at 13,000 rpm in a microcentrifuge for 5 min at 4°C. Supernatants were incubated with loading buffer for 30 min at room temperature, and proteins were analyzed by SDS–PAGE and Western blot.
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