IDENTIFICATION OF RETRIEVAL-TRAFFICKING OF THE HUMAN ETHER A-GO-GO-RELATED GENE CHANNEL

By

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ABSTRACT

The human ether-a-go-go related gene (hERG) encodes the pore-forming subunit of the rapidly activating delayed rectifier potassium channel (I_{Kr}). A reduction in the hERG current causes long QT syndrome, predisposing affected individuals to a high risk of cardiac arrhythmias and sudden death. We previously reported that hERG channels in the plasma membrane undergo internalization under low K\(^+\) conditions. While studies have characterized the synthesis and degradation pathways that maintain the homeostasis of hERG density in the plasma membrane, whether internalization occurs under normal K\(^+\) conditions and whether internalized channels can be recycled to the plasma membrane are not addressed. Using patch clamp, Western blot and immunocytochemical analyses, we studied the retrieval trafficking of internalized hERG channels to the plasma membrane. Our data demonstrated that an enhanced internalization is accompanied by an increased rate of recovery of hERG channels to the plasma membrane. The increased recovery rate is not due to enhanced protein synthesis, since hERG mRNA expression level was not altered by low K\(^+\) exposure, and the increased recovery was not affected by the inhibition of protein synthesis using cycloheximide. Disrupting the trans-Golgi network with brefeldin A (BFA) blocked not only hERG maturation, but also the retrieval trafficking. Given these findings, we were interested to identify which Rab GTPase proteins were involved in this recycling pathway, since these proteins are known to regulate trafficking. Our study demonstrated that GTPase Rab11, but not Rab4, is involved in the retrieval trafficking of hERG channels. Furthermore, interfering with Rab11 function not only delayed hERG recovery after exposure to low K\(^+\) medium, but led to decreased hERG expression and function under normal culture conditions. We
conclude that the retrieval trafficking plays an important role in the homeostasis of membrane-bound hERG channels.
CO-AUTHORSHIP

Mr. Jun Guo and Wentao Li collected majority of the patch clamp data of hERG channels for Figures 6, 7, 10, 16, 19, and 21. Ms. Tonghua Yang assisted with Figure 18 for the confocal imaging experiment, and Mr. Jun Guo assisted in the immunofluorescence experiment for Figure 12.
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0 mM K⁺ does not increase hERG protein synthesis.

Internalized hERG channels can recycle back to the plasma membrane.

Glycan-modification occurs during internalization and retrieval trafficking of hERG channels.

Rab11 interacts with hERG.

Rab11 plays a role in the recovery of the hERG current from 0 mM K⁺.

Rab11 plays a role in the recovery of the mature hERG expression from 0 mM K⁺.

Recycling of hERG channels occurs under endogenous conditions.

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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ATPase</td>
<td>Adenosine Triphosphate Hydrolase</td>
</tr>
<tr>
<td>BFA</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>Co-IP</td>
<td>Co-immunoprecipitation</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic Fibrosis Transmembrane Conductance Regulator</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential Interference Contrast</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<tr>
<td>EE</td>
<td>Early Endosome</td>
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<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-Activating Protein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanine Diphosphate</td>
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<tr>
<td>GEF</td>
<td>GDP/GTP Exchange Factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine Triphosphate Hydrolase</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HEK</td>
<td>Human Embryonic Kidney</td>
</tr>
<tr>
<td>HERG</td>
<td>Human Ether-a-go-go Related Gene</td>
</tr>
<tr>
<td>I_{hERG}</td>
<td>hERG Current</td>
</tr>
<tr>
<td>I_{Kr}</td>
<td>Rapidly Activating Delayed Rectifier Potassium Current</td>
</tr>
<tr>
<td>I_{Ks}</td>
<td>Slowly Activating Delayed Rectifier Potassium Current</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>$I_{so}$</td>
<td>Transient Outward Potassium Current</td>
</tr>
<tr>
<td>$K_v$</td>
<td>Voltage-gated potassium channel</td>
</tr>
<tr>
<td>LE</td>
<td>Late Endosome</td>
</tr>
<tr>
<td>LQTS</td>
<td>Long QT Syndrome</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Media</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>PAS</td>
<td>Per-Arnt-Sim</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PK</td>
<td>Proteinase K</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Difluoride</td>
</tr>
<tr>
<td>RE</td>
<td>Recycling Endosome</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase - Polymerase Chain Reaction</td>
</tr>
<tr>
<td>TdP</td>
<td>Torsades de Pointes</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
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Long QT Syndrome

Long QT syndrome (LQTS) is a heart condition that can predispose individuals to cardiac arrhythmia, syncope, and Torsade de Pointes (TdP) which may lead to sudden death (Schwartz et al., 1993; Vincent et al., 1992) (Figure 1). LQTS is estimated to affect 1 in 5000-10000 people worldwide (Vincent, 2002). This condition is caused by delayed repolarization during the action potential of ventricular cardiomyocytes, and is typically defined as any QT interval greater than 450 ms in males, and 470 ms in females (Vincent, 2002) (Figure 1). The name long QT syndrome is derived from the prolonged QT interval observed from electrocardiogram (ECG) recordings. An ECG takes surface recordings of electrical activities that occur in the heart starting with the initial electrical impulse originating from pacemaker cells that are collected at the sinoatrial node, and the conduction throughout the rest of the heart. The ECG recording of a single normal cardiac cycle is shown as a PQRST wave: the P wave is caused by atrial depolarization, following which the electrical activity spreads towards the two atrial chambers, the QRS complex represents ventricular depolarization, and the T wave displays the repolarization of the ventricles.

The rapidly ($I_{Kr}$) and slowly ($I_{Ks}$) activating delayed rectifier potassium channels are responsible for the repolarization phase during the cardiac cycle (Barhanin et al., 1996; Sanguinetti et al., 1995). The first complete description of LQTS was given by Jervell and Lange-Nielsen in 1957, who characterized it with deafness that was inherited as an autosomal recessive trait, causing sudden death – this disease was subsequently
Figure 1. Reductions in $I_{hERG}$ lead to prolonged ventricular action potentials and LQTS.

A: A comparison between normal human ventricular action potential (left) and a prolonged action potential as a result of reduced hERG current. B: Normal QRST wave (left) compared to a long QT interval. C: Diagram of an ECG tracing showing Torsade de Pointes (TdP) arrhythmia. Modified from Sanguinetti & Firouzi, (2006).
named the Jervell-Nielsen syndrome (Jervell & Lange-Nielsen, 1957). In 1964, Romano and Ward described some families with the same signs as characterized by the Jervell-Nielsen syndrome that was inherited as an autosomal dominant trait, but without deafness, and named it the Romano-Ward syndrome (Romano et al., 1963; Ward, 1964). The difference in signs that manifested from LQTS established the idea that there were variations of LQTS, which is currently found to arise from various mutations. There are presently up to 13 different classifications of LQTS: LQT1 – LQT13, with each type arising from mutations of different genes. Of the different types, LQT1 – LQT3 are the most common; mutations in \( \text{KCNQ1} \) that encodes the \( I_{\text{Ks}} \) lead to LQT1, \( \text{KCNH2} \) that encodes the \( I_{\text{Kr}} \) lead to LQT2, and \( \text{SCN5A} \) that encodes the sodium channel, \( \text{Na}, 1.5 \), lead to LQT3 (Vincent, 2002). More typically, these mutations result in a loss of functions of channels that are responsible for repolarization (Anderson et al., 2006). Aside from inheriting mutations, LQTS can also arise from drug interactions and exposure to certain conditions such as hypokalemia (Keating & Sanguinetti, 2001; Sanguinetti & Tristani-Firouzi, 2006).

**Action Potentials Contributing to the PQRST Wave**

Individuals with LQTS may not show any signs or symptoms, but the long QT interval will manifest on an ECG recording, reflecting prolonged ventricular repolarization. This repolarization is contributed by the action potentials of ventricular myocytes that are divided into five phases. At phase 0, depolarization of the cells is caused by the inward sodium current. At phase 1, repolarization follows quickly, but only for a brief period due to the transient outward potassium current (\( I_{\text{o}} \)). A plateau in the action potential follows in phase 2 as a result of competing inward flow of calcium via L-
type calcium channels and outward flow of potassium current. Phase 3 repolarization is mediated by \( I_{Kr} \) and \( I_{Ks} \) to restore the resting membrane potential as characterized by phase 4. Prolongation of phase 3 in ventricular myocytes leads to long QT intervals; this phase is regulated by hERG channels that are responsible for \( I_{Kr} \) (Sanguinetti et al., 1995) (Figure 1).

**hERG Channel – Its Role in LQTS**

The *human ether-a-go-go-related gene* (*hERG* or recently denoted as *KCNH2*) on chromosome 7 encodes the pore forming \( \alpha \)-subunit of the rapidly activating delayed rectifier potassium channels (Curran et al., 1995; Sanguinetti et al., 1995; Trudeau et al., 1995). As well as being expressed in ventricular cardiomyocytes, hERG channels are also found in smooth muscle cells, neural tissues, and tumour cells, though, their function is best understood in the heart (Chiesa et al., 1997; Smith et al., 2002; Warmke & Ganetzky, 1994). Inherited mutations or channel blockade, ironically by anti-arrhythmic drugs, can result in familial or acquired LQTS, respectively (Keating & Sanguinetti, 2001; Sanguinetti & Tristani-Firouzi, 2006). To date, at least 200 mutations have been discovered to result in hERG channelopathies, resulting in the disruption of proper folding of the protein, trafficking to the plasmalemma, and altered hERG gating kinetics that renders it non-functional or dysfunctional (Delisle et al., 2004; Furutani et al., 1999; Kagan et al., 2000; Sanguinetti et al., 1996).

**hERG Channel Structure**

The hERG channel, a member of the voltage-gated potassium (VGK) channel superfamily, is a 1159-amino acid tetrameric protein of identical \( \alpha \)-subunits, with each subunit containing six \( \alpha \)-transmembrane domains, S1-S6, with the N-terminal and the C-
terminal tails located intracellularly (Warmke & Ganetzky, 1994) (Figure 2). The N-terminal tail is shown to be related to the Per-Arnt-Sim (PAS) domain, a protein domain that normally functions as a signal sensor, and is involved in channel deactivation (Morais Cabral et al., 1998). The C-terminal tail contains a cyclic nucleotide binding domain which when mutated, is found to result in defective endoplasmic reticulum (ER) processing and export (Akhavan et al., 2005). Like other VGK channels, S1-S4 acts as the membrane-voltage sensor, while S5 and S6 constitutes the potassium-selective pore; the hERG channel contains a positively charged S4 transmembrane domains that acts as the primary voltage-sensor, containing an arginine or lysine in every third position, that detects depolarized potentials and moves outward (Swartz, 2004) (Figure 2). Like other VGK channels, the hERG channel has a conserved sequence at the potassium-selectivity filter on the extracellular side of the S5-S6 pore at the carboxy-terminal end, with the sequence Ser-Val-Gly-Phe-Gly (Doyle et al., 1998).

**hERG Gating Kinetics**

As a VGK channel, the gating kinetics of hERG channels can be characterized into three distinct phases: closed, open, and inactivated, which are dependent on the membrane potential (Vandenberg et al., 2012) (Figure 3). These distinct phases occur as a result of conformational changes. Although a member of the VGK channel superfamily, the hERG channel has unique gating kinetics; the voltage-dependent inactivation phase is faster than the voltage-dependent activation phase (Bett et al., 2011). As a result, the hERG channel current is much more active during the repolarization phase of ventricular action potentials. The hERG channel is in its closed state at negative membrane potential.
Figure 2. Structure of the hERG channel. Cartoon depiction of the hERG structure. A. A front view of the single hERG subunit. The hERG subunit is composed of the per-arnt-sim (PAS) domain (1–137), proximal N-terminus (138–406), voltage sensor (407–545), pore domain (545–665), C-linker (666–742), cyclic nucleotide binding domain (cNBD; 742–844), distal C-terminus (845–1159). B. A top view of the hERG channel assembled as a tetramer. Modified from Perrin et al. (2008).
Figure 3. Gating kinetics of the hERG channel. The three distinct phases as a result of conformational changes in the hERG channel are closed, open, and inactivated, which are dependent on the membrane potential. Modified from Vandenberg et al. (2012).
When the cell membrane potential moves toward zero during depolarization, hERG channels are opened to allow the diffusion of $K^+$ ions outwards as a result of the electrochemical gradient. As depolarization continues, hERG channels begin to enter a non-conducting inactivated phase. Finally, as the cell membrane potential repolarizes, channels de-inactivate quickly to allow a peak conductance of $K^+$ flow outwards.

Potassium channels are characterized by either C-type inactivation, a conformational change that results in the collapse of the selectivity filter that is mediated by the carboxyl terminal, or the N-type inactivation, where a “ball” attached to a “chain” from the amino terminal blocks the pore, both of which prevent ion flow through the channel (Vandenberg et al., 2012). Studies on mutations in the S6 domain of the hERG channel suggest that its inactivation is C-type (Smith et al., 1996).

hERG Channel Expression

The hERG protein is synthesized in the ER where it is subsequently modified by glycosylation at asparagine on N598, resulting in the immature 135 kDa form from a 132 kDa precursor (Gong et al., 2002). Complex folding of the protein involves the recruitment of chaperones that reside in the ER or the cytosol. The function of these chaperones is to provide a quality control during the synthesis and folding of the proteins; hsp70 and hsp90 promote trafficking of properly folded hERG channels and retain misfolded hERG proteins (Ficker et al., 2003). These chaperones recognize motif determinants on the hERG protein that signal whether it will be trafficked out of the ER for further processing or held back for degradation. The hERG protein has multiple ER retention motifs, the Arg-X-Arg (RXR) motif, and the diacidic motif to signal for export out of the ER for further modification (Zerangue et al., 1999; Mikosch & Homann,
2009). When folded correctly, these ER retention motifs are presumed to be hidden from the cellular environment, but when the protein is misfolded, exposed ER retention motifs allow quality control chaperones to recognize and bind onto the defective protein in order to retain it in the ER (Zerangue et al., 1999). Following folding and modifications in the ER, the chaperones dissociate and the immature hERG protein is trafficked to the Golgi apparatus for further complex glycosylation to become the fully-glycosylated mature 155 kDa form (Gong et al., 2002). The hERG channel contains two consensus sites for N-linked glycosylation: N598 and N629 (Petrecca et al., 1999). A study using site-directed mutagenesis showed that although glycosylation at the N598 site is not necessary for proper trafficking of functional hERG channels to the plasmalemma, it decreases the protein turnover rate, and while N629 does not undergo N-linked glycosylation, mutations to this site produced defective trafficking of the protein out of the ER (Gong et al., 2002). After full glycosylation, the hERG channel is transported to the surface membrane to be inserted, with a turnover rate of WT hERG channels on the plasmalemma of about 10 hours (Delisle et al., 2009). When channels are internalized into endocytic vesicles, they may be targeted for degradation by the proteasomal pathway if the hERG protein is tagged by a polyubiquitin chain, whereas monoubiquitination of the protein results in degradation by the lyosomal pathway (Chapman et al., 2005; Gong et al., 2005). Certain environmental conditions have been discovered to affect the half-life of the channel on the cell surface; for example, cells exposed to low extracellular K+ concentrations have been shown to affect surface hERG channels by inducing rapid internalization via caveolin-dependent endocytosis and increased degradation by ubiquitination (Guo et al., 2009; Massaeli et al., 2010a; Massaeli et al., 2010b)
Hypokalemia-Induced hERG Internalization

The hERG protein is unusually susceptible to dysfunction induced by drugs, and conditions such as hypokalemia that can lead to acquired LQTS. hERG-HEK cells that had chronic exposure to hypokalemic conditions, a reduction in extracellular $K^+$ concentration of below 3.5 mM, showed decreased density of hERG channels in the plasma membrane; patch clamp recordings of cells treated in 0 mM $K^+$ conditions for 6 h had no hERG current, and confocal images suggested that the molecular mechanism that led to a reduction in plasma membrane hERG density was accelerated internalization rate of the hERG protein (Guo et al., 2009; Massaeli et al., 2010a).

Small GTPase Rab Proteins

Maintenance of normal QT intervals is controlled by the homeostasis of functional hERG channels with an equilibrium between forward trafficking of newly synthesized channels, and the degradation of old channels to keep a balanced population of membrane protein. The balance of trafficking hERG channels into or out of the plasmalemma is mediated by transport vesicles. Rab proteins, small guanine triphosphate hydrolases (GTPases) that belong to the Ras superfamily, play a role in maintaining the balance of surface protein density by regulating budding vesicular formation, transport of vesicles along actin and tubulin networks, tethering vesicles to their target, then fusion at their final destination (Novick & Zerial, 1997). In the human genome, there exist at least 60 different gene coding Rab isoforms (Bock et al., 2001). Some classes of these small GTPases are expressed ubiquitously while others may only be predominantly found in select tissues. These small proteins exist in two conformations; when bound to guanine diphosphate (GDP), they are inactive, but through a catalytic reaction by the GDP/GTP
exchange factor (GEF), whereby the nucleotide exchange converts them to the GTP-bound conformation, they are activated to interact with downstream effector proteins, then cycled back to their GDP-bound inactive form through GTP hydrolysis catalyzed by the GTPase-activating protein (GAP) (Stenmark et al., 1994). The recruitment of downstream effector protein allows Rab GTPases to traffic cytosolic or internalized proteins to different intracellular compartments (Zerial & McBride, 2001).

Rab Protein Structure

Despite the various classifications of the Rab proteins, many subfamilies of Rab GTPases have similar sequences and overlapping functions. The most conserved region amongst the Rab GTPases is the guanine-nucleotide binding domain, but they differ in their distinct carboxyl termini, which are suggested to determine their different subcellular localization (Chavrier et al., 1991). X-ray crystallography has shed light on the conserved structure of the Rab proteins; they share a common six-stranded β-sheet of five parallel strands and one anti-parallel strand, flanked by five α-helices (Stenmark & Olkkonen, 2001). The catalytic site that remains open for guanine nucleotide and magnesium binding, as well as GTP hydrolysis, is the five loops that attach the α-helices and β-sheets (Stenmark & Olkkonen, 2001).

Rab Proteins involved in Recycling

Although not well understood, it is known that internalized molecules are not subjected to strict degradation, as the intracellular molecular mechanism may involve recycling. Internalization begins via clathrin or non-clathrin coated pits that allow membrane proteins to invaginate and pinch off (Mukherjee et al., 1997). When proteins are internalized from the cell surface, these budded vesicles fuse with early endosomes
which can then either be subsequently targeted for degradation when tagged with ubiquitin, a 76 amino acid protein, for transport to late endosomes (LE) and lysosomes, or stored within a separate perinuclear compartment, the recycling endosome (RE), for future recycling back to the plasmalemma (Maxfield & McGraw, 2004). Different endosomal sorting compartments have their own specialized luminal pH levels; for example, LE-containing molecules targeted for degradation become more acidic with the accumulation of acid hydrolases, while RE that are found in the periphery of the intracellular space are less acidic (Dunn et al., 1989). The EE is a general term to describe two different compartments; the sorting endosome and the endocytic recycling compartment (Maxfield & McGraw, 2004). As its name suggests, the sorting endosomes are responsible for delivering molecules to their appropriate destinations. The acidic environment in the lumen of these sorting endosomes allow for ligands to mix within the compartment (Mukherjee et al., 1997). However, most transmembrane proteins are removed from sorting endosomes and trafficked to the endocytic recycling compartment before sorting endosomes mature into late endosomes by pinching off of narrow-diameter tubules (Dunn et al., 1989). Recycling may occur directly from sorting endosomes to the plasmalemma, or channels may be stored in the endocytic recycling compartment for a longer period. Direct protein recycling can have a $t_{1/2}$ of 6 minutes (Sheff et al., 1999). The exact proportion that undergoes recycling via the indirect or direct pathway is not known due to technical difficulties in analyzing such a rapid process. Because the endocytic recycling compartment is a long-lived space, transportation of transmembrane proteins back to the plasmalemma requires transport intermediates, such as the requirement of vesicle formation. The small GTPases Rab 4 and Rab11 are localized in
the EE and recycling endosome (RE), respectively, and are identified to be responsible for recycling molecules back to the plasmalemma, with Rab11 being involved specifically at the level of the endocytic recycling compartment for the indirect recycling pathway (Gruenberg & Maxfield, 1995) (Figure 4).
Figure 4. A cartoon depiction of intracellular vesicle transport and the localization of different Rab GTPases. Rab GTPases mediate the transport of proteins throughout many stages starting from the biosynthetic trafficking to the internalization of proteins ready for degradation. Molecules internalized may be stored in early endosomes (EE) and recycled back via Rab4 or they may be stored in recycling endosomes (RE) for future re-processing back to the surface via Rab11. Modified from Stenmark and Olkkonen, (2001).
Hypothesis and Study Objectives

Much of what we currently know about the processing of hERG channels from their synthesis to their intracellular modification and degradation was derived from work on the cystic fibrosis transmembrane conductance regulator (CFTR). Thus, we now recognize the significance of regulatory GTPases on hERG channel trafficking, and the importance of their function on Golgi processing and trafficking to the cell membrane (Delisle et al., 2009). Although many studies have focused on forward trafficking and the degradation pathway that regulate hERG channel density in the plasma membrane, whether hERG channels are recycled back to the plasma membrane remains unclear. Recent accumulating evidence has directed our attention towards understanding the trafficking machinery of hERG channels as 90% of missense mutations of hERG result in defective trafficking, attracting the idea of manipulating this recycling machinery to improve LQTS recovery (Delisle et al., 2009). Our lab has recently demonstrated that hypokalemia induces hERG channel internalization (Guo et al., 2009; Massaeli et al., 2010). The hypothesis of my project is that internalized hERG channels can recycle back to the plasma membrane. Accelerated internalization induced by hypokalemia provides an excellent opportunity to study the recycling pathway of hERG channels by analyzing their recovery rate. Understanding the trafficking pathway of hERG channels may lead to the development of preventative measures and novel treatment of long QT syndrome.

The objectives of this study to test my hypothesis are to:

1. Identify whether hERG channels are regulated by a recycling pathway.
2. Characterize the recycling pathway that hERG channels utilize.
CHAPTER 2: MATERIALS AND METHODS

Molecular Biology

A human embryonic kidney (HEK) 293 cell line stably expressing hERG channels (hERG-HEK cells) was provided by Dr. Craig January (University of Wisconsin-Madison); hERG cDNA was provided by Dr. Gail Robertson (University of Wisconsin-Madison). The GFP-Rab11, GFP-Rab11 dominant-negative mutant S25N GFP-Rab11, GFP-Rab4, and GFP Rab4 dominant-negative mutant N121I GFP-Rab4 plasmids were obtained from Addgene, as well as Dr. Terry Hébert (McGill University, Montreal, ON, Canada). HEK 293 cells were cultured in Minimum Essential Medium (MEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen). Lipofectamine 2000 (Invitrogen) was used for transfecting plasmids into HEK 293 cells. For immunofluorescence staining of the cell-surface hERG channels in live cells, a HA-epitope tag of the sequence 436TEEGPPATNSEHYDPYDVPDYAVTFEECGY (bold: insertion; underlined: HA epitope) was inserted into the extracellular S1-S2 loop of hERG channels to generate hERG-HAex via PCR using the overlap extension method (Ho et al., 1989). The hERG-HAex was transfected into HEK 293 cells and a stable hERG-HAex cell line (hERG-HAex-HEK) was created using an aminoglycoside antibiotic, G418 (Invitrogen), for selection (1 mg/ml) and maintenance (0.4 mg/ml). As reported previously by others as well as our team, inserting HA into hERG in this manner does not change the electrophysiological or trafficking properties of hERG channels (Ficker et al., 2003; Guo et al., 2007).

Western Blot Analysis

hERG-HEK cells of 80% confluence were harvested from 35-mm dish per group
with ice-cold scrapers following three washes with ice-cold phosphate buffered saline (PBS). Cells were centrifuged at 1,000 R.P.M. (J-20 rotor) for 4 minutes at 4°C. The supernatant of PBS was aspirated and the isolated pellet of cells was immersed in a lysis buffer containing 1% protease inhibitor cocktail (Sigma-Aldrich). The pellet of cells were given a fast lysis sonication and centrifuged at 10,000 R.P.M. for 10 minutes. Whole-cell lysates were collected in the supernatant for subsequent measurement of protein concentration using a modified Lowry assay. A protein standard of Bovine Serum Albumin (BSA) (Sigma-Aldrich) solution was used for comparison of lysate protein concentration. Protein samples were made by the addition of double distilled water and 5× Laemmli sample loading buffer containing 5% β-mercaptoethanol, followed by a 5-minute boil. Lysate samples were separated on 8.0% polyacrylamide gels and electroblotted overnight at 4°C onto a polyvinylidene difluoride (PVDF) membrane.

Membranes were blocked for 1 h using 5% skim milk in 0.1% Tween 20 in Tris-buffered saline (TBS). Blocked membranes were immunoblotted for 1 h using appropriate primary antibodies: rabbit anti-Kv11.1 (Sigma-Aldrich) and goat anti-hERG (C-20) (Santa Cruz Biotechnology) to detect hERG, mouse anti-actin (Sigma-Aldrich) to detect actin, rabbit anti-GFP (Sigma-Aldrich) to detect GFP-tagged Rab4 and Rab11 expression.

Immunoblotted membranes were then incubated with corresponding horseradish peroxidase-conjugated secondary antibodies. Protein signals were detected using an enhanced luminol-based chemiluminescent detection kit (GE Healthcare). The Precision Plus Protein Dual Color Standard was used as the protein ladder (Bio-Rad). For quantification of Western blot data, intensities of proteins of interest in each gel were first normalized to their respective actin intensities; the normalized intensities were then
compared with the intensity from control cells and expressed as relative values to their controls.

**Co-immunoprecipitation (co-IP)**

Whole-cell proteins (0.5 mg) were incubated with an appropriate primary antibody (anti-GFP antibody or anti-GAPDH for control) targeting a specific protein at 4°C overnight, and then precipitated with 40 μl protein A/G PLUS Agarose beads (Santa Cruz Biotechnology) at 4°C for 4 h. The immunoprecipitate was washed 3 times with radioimmunoprecipitation assay (RIPA) lysis buffer. 2× Laemmli sample loading buffer was added to the pelleted immunoprecipitate and boiled for 5 min. The sample was centrifuged at 20,000 × g for 5 min, and the supernatant was collected for Western blot analysis to detect proteins that are associated with the pull-down protein.

**Cell-Surface Protein Assay**

For the analysis of cell-surface proteins, a Cell Surface Protein Isolation Kit (Biotinylation kit, Pierce) was used. hERG-HEK cells were cultured in 100-mm dishes and grown to 90% confluence. The cells were labeled with 10 ml of the membrane-impermeant biotinylating reagent, Sulfo-NHS-SS-biotin, for 30 min at 4°C. The quenching solution (0.5 ml) was then added to stop the reaction. Cells were then lysed with 0.5 ml of lysis buffer containing 1% protease inhibitor cocktail. After centrifugation at 10,000 × g for 2 min at 4°C, the cell lysate was precipitated with Immobilized NeutrAvidin Gel (agarose beads). The bound proteins were eluted by incubating the resin in a Tris buffer (62.5 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol) containing 50 mM dithiothreitol (DTT). Eluted cell surface protein was then analyzed using Western blot analysis.
Cell-Surface Protein Digestion

In order to eliminate the 155-kDa hERG protein on the cell surface, hERG-HEK cells cultured in 5 or 0 mM K⁺ MEM were treated with Proteinase K. Specifically, intact live cells were washed with PBS and treated with 200 μg/ml Proteinase K (Sigma-Aldrich) in a physiological buffer (10 mM HEPES, 150 mM NaCl, and 2 mM CaCl₂, pH 7.4) at 37°C for 20 min to cleave cell surface proteins. The reaction was terminated by adding ice-cold PBS containing 6 mM phenylmethylsulfonyl fluoride and 25 mM ethylenediaminetetraacetic acid (EDTA).

Trypsin Assay

Depending on the folding configuration of a protein, some may be more susceptible to enzyme digestion. To examine the sensitivity of hERG proteins to trypsin digestion, samples of 20 μg whole-cell proteins from hERG-HEK cells cultured in 5 or 0 mM K⁺ MEM for 12 h were treated with various concentrations of trypsin (~10,000 benzoyl-L-arginine ethyl ester units/mg protein; Sigma-Aldrich) for 5 min at room temperature. The reaction was stopped with lima bean trypsin inhibitor (Sigma-Aldrich). Digested samples were subsequently analyzed using Western blot analysis.

Endocytosis Assay

Biotin was used to track the endocytosed hERG channels. hERG-HEK cells cultured in 5 or 0 mM K⁺ MEM for 12 h were cultured in 0 mM K⁺ Tyrode solution with biotin (0.25 mg/ml) for 2 h to allow internalization of biotinylated cell-surface hERG channels. Cells were then cultured in 0 mM K⁺ MEM for an additional 4 h. Whole-cell protein lysates were collected, and biotinylated proteins were isolated using NeutrAvidin beads and analyzed using Western blot analysis.
Immunofluorescence Microscopy

To assay co-localization between hERG and Rab11, hERG-HEK cells were prepared by growing them on glass coverslips for 24 h. Cells were then cultured in 5 or 0 mM K\(^+\) MEM for 3 h, fixed with 4% paraformaldehyde in 1×PBS for 15 min, permeabilized with 0.1% Triton X-100 in 1×PBS for 10 min, and blocked with 5% bovine serum albumin (BSA) (Sigma-Aldrich) in 1×PBS for 1 h. hERG channels were stained with goat anti-HERG primary (C-20) (Santa Cruz Biotechnology) and Alexa Fluor 488-conjugated donkey anti-goat secondary antibodies (Invitrogen). Endogenous Rab11 was detected with rabbit anti-Rab11 primary (Santa Cruz Biotechnology) and Alexa Fluor 594-conjugated donkey anti-rabbit secondary antibodies (Invitrogen). Antibodies were diluted in 1:50 in 2.5% BSA with 1×PBS. Images were acquired using a Leica TCS SP2 Multi Photon confocal microscope (Leica, Germany).

To visualize the retrieval trafficking of hERG channels, hERG-HAex-HEK cells grown for 24 h on glass bottom plates were incubated with anti-HA-FITC antibody (Abcam) at 4°C for 20 min. Unbound antibody was then washed away with PBS. Cells were cultured in 0 mM K\(^+\) MEM at 37°C for 4 h to induce hERG channel endocytosis. After taking images, the cells were then cultured under normal (5 mM K\(^+\)) conditions for an additional 4 h to observe recycling of the labeled channels. Images were acquired using a Zeiss Observer.Z1 inverted fluorescence microscope (Zeiss, Germany).

To examine the retrieval trafficking of hERG channels in cells under normal culture conditions, hERG-HAex-HEK cells grown on glass cover slips for 24 h were labeled with mouse anti-HA primary antibody (Sigma-Aldrich) for 30 min at 4°C. Cells were incubated for 2 h to allow internalization of labeled HA-hERG under normal culture
conditions. Un-internalized labeled HA-hERG channels on the membrane were then stained with Alexa Fluor 594-conjugated donkey anti-mouse antibodies (Invitrogen) for 30 min. Cells were then cultured for an additional 2 h in normal conditions to allow previously internalized labeled channels to recycle back to the plasma membrane. Recycled channels were then labeled with Alexa Fluor 488-conjugated goat anti-mouse antibodies (Invitrogen) for 30 min. Cells were fixed at various stages in 4% paraformaldehyde in 1×PBS for 15 min. Images were acquired using a Leica TCS SP2 Multi Photon confocal microscope.

**Reverse Transcription – Polymerase Chain Reaction**

Total RNA was extracted from hERG-HEK cells cultured in 5 or 0 mM K⁺ MEM using a Total RNA Mini Kit as per manufacturer’s instructions (Geneaid Biotech Ltd., Taiwan). cDNA was generated using 1 µg of RNA, 2 µl of polyN hexamers (100 µmol/L, Eurofins MWG Operon, Germany), nuclease-free water, and an Omniscript Reverse Transcription Kit (QIAGEN) to make a total volume of 20 µl. To amplify hERG cDNA, 1 µl of cDNA was added to a PCR reaction tube containing: 10 µl of 2×PCR Master Mix (Thermo Scientific), 1 µl of the forward/reverse hERG primer mixture, and 8 µl of nuclease-free water to reach a final volume of 20 µl. A 4 min incubation at 94°C served to activate the Taq DNA Polymerase contained in the 2× PCR Master Mix. The PCR was conducted with 30 cycles of 94°C for 20 s, 60°C for 20 s, and 72°C for 20 s. A series of different cycles were run and 30 cycles was selected as the optimal number for primer amplification. The last cycle was followed by a 5-minute holding at 72°C. Primers for hERG cDNA amplification were: forward, 5’-TCGCCTTCTACCGGAAAGAT-3’ and reverse, 5’-CTCCATCACCACCCTCGAAATTG-3’. β-actin (internal control) primers
were: forward, 5’-CATCCTGCGTCTGGACCT-3’ and reverse, 5’-TAATGTCACGCACGATTTCC-3’. The amplified DNA was loaded with a 6× gel loading dye (New Englands Biolab) and electrophoresed on a 1% agarose gel that contained SYBR Safe DNA gel stain (Invitrogen), and visualized with against a 50bp DNA ladder (Invitrogen) under an ultraviolet transilluminator.

Whole-Cell Voltage Clamp Recording

The hERG channel current (I_{hERG}) was recorded using the whole-cell patch clamp method. The bath solution contained (in mM) 135 NaCl, 5 KCl, 10 HEPES, 10 glucose, 1 MgCl₂, and 2 CaCl₂ (pH 7.4 with NaOH). The internal pipette solution consisted of (in mM) 135 KCl, 5 EGTA, 1 MgCl₂, and 10 HEPES (pH 7.2 with KOH). I_{hERG} was recorded by depolarizing steps to voltages between −70 and 70 mV in 10 mV increments from a holding potential of −80 mV. A repolarizing step to −50 mV was used to record the tail currents (Figure 5). Tail currents were used in the measurement of all I_{hERG} for consistency in current recording due to the saturation in the conductance of I_{hERG} following recovery from inactivation. Patch clamp experiments were conducted at room temperature (22 ± 1°C). Plasmid transfections were performed with either GFP-tagged plasmid or co-transfected with a GFP plasmid. Thus, only GFP positive cells were recorded. Clampex 10.3 was the software used to obtain whole cell patch clamp data. Sampling frequency for I_{hERG} recording was 1 kHz. Filtering during I_{hERG} recording was 5 kHz. Prior to perforation of the cell, pipette resistance and capacitance were compensated for using the amplifier. Series resistance and capacitance was compensated for using the amplifier upon reaching a Gigaseal resistance with the cell. Patch clamp data was analyzed using Clampfit 10.3 software to measure peak tail current of I_{hERG}.
Figure 5. *Step protocol for I_{hERG} recording*. A. Cells underwent depolarizing voltages between -70 and +70 mV in 10-mV increments from a holding potential of -80 mV. B. A peak tail current elicited by the voltage protocol in A. C. A current-voltage relationship for peak I_{hERG}. D. A conductance-voltage relationship of I_{hERG}. Peak tail current was used for all I_{hERG} recording for consistency due to plateau in conductance following recovery from voltage dependent hERG channel inactivation.
Densitometry

Densitometry was performed on Western blot images. Developed Western blot films were scanned using the Epson V700 Scanner. Images were converted to gray scale using Adobe Photoshop. The density of each individual band was quantified. Measured densities of samples from whole-cell lysates were normalized to their respective actin loading control.

Statistical Analysis

All data are expressed as the mean ± the standard error of the mean (S.E.M). A one-way ANOVA or 2-tailed Student’s t-test was used to determine statistical significance between the control and test groups. A P-value of 0.05 or less was considered significant.
CHAPTER 3: RESULTS

Proteinase K and 0 mM K⁺ MEM clear functional surface membrane hERG channels

Our lab demonstrated that 0 mM K⁺ induces accelerated hERG internalization (Guo et al., 2009; Massaeli et al., 2010a). This phenomenon creates an excellent opportunity to study whether hERG is regulated by a recycling pathway, by comparing the recovery rate of depleted channels from cells destroyed by proteases versus cells treated under 0 mM K⁺ conditions. In a Western blot analysis, WT hERG display two bands: the 135 kDa band represents the immature core-glycosylated channels, and the 155 kDa band represents the fully-glycosylated form (Figure 6). Compared to cells cultured in 5 mM K⁺ MEM, cells cultured in 0 mM K⁺ MEM for 6 h yielded essentially no 155-kDa band with an increase in the 135-kDa band. To demonstrate that the 155-kDa band is localized in the plasma membrane while the 135-kDa band is localized intracellularly, a membrane impermeable serine protease, Proteinase K (200 μg/ml), was used to cleave surface hERG protein. Figure 6 shows that the 155-kDa band was cleared accompanied by an appearance of a cleavage product of an expected 65-kDa since Proteinase K cuts the extracellular pore-linker on the hERG channel, while the 135-kDa band remained unaffected. Peak tail current analysis of $I_{\text{hERG}}$ following treatment of Proteinase K or/and 0 mM K⁺ conditions show complete reductions.

Recovery rate of $I_{\text{hERG}}$ is faster in cells cultured in 0 compared to 5 mM K⁺ following Proteinase K digestion of surface membrane hERG

To study whether internalized hERG channels can traffic back to the plasma membrane, the recovery rate of hERG channels following culture in 0 mM K⁺ conditions was compared to cells treated in 5 mM K⁺ conditions. Following a 12-h culture, both
**Figure 6.** Proteinase K and 0 mM K$^+$ MEM clear functional surface membrane hERG channels: effects of Proteinase K and 0 mM K$^+$ MEM on hERG expression and I$_{hERG}$. Representative Western blot image and I$_{hERG}$ of hERG-HEK cells cultured in 5 (control, Ctl) or 0 mM K$^+$ MEM for 6 h with or without Proteinase K digestion (PK, 200 µg/ml). The numbers in parentheses above each bar indicate the number of cells tested from three independent trials. ***, $P < 0.01$ versus 5 K$^+$. 
groups were given subsequent Proteinase K (200 µg/ml) treatment to clear cell surface membrane hERG, and cultured back in 5 mM K⁺ MEM. Figure 7 shows that recovery of $I_{hERG}$ at different time points of culture in 5 mM K⁺ MEM after Proteinase K treatment from 0 mM K⁺ pre-cultured cells recovered much faster than that in 5 mM K⁺ pre-cultured cells. Since the greatest difference in peak tail current was greatest between 4-8 h after hERG recovery, subsequent Western blot and patch-clamp experiments were conducted with 6 h recovery. Consistent with the increased recovery in $I_{hERG}$ observed in 0 mM K⁺ pre-cultured cells, the recovery of the mature 155-kDa band was greater in 0 mM K⁺ pre-cultured cells compared to 5 mM K⁺ pre-cultured cells (Figure 8). This could be a result of two possibilities: following culture in 0 mM K⁺, an enlarged reservoir of internalized hERG channels account for the increase in recovery, or 0 mM K⁺ conditions are increasing hERG protein synthesis.

0 mM K⁺ does not increase hERG protein synthesis

To study whether the previous observation was due to recycling or increased synthesis, we first analyzed hERG transcription in hERG-HEK cells cultured in 5 or 0 mM K⁺ MEM for 12 hours. Following incubation, hERG mRNA was extracted for transcriptional profiling using RT-PCR. Figure 9 shows no difference in amplified hERG primer products between cells cultured in 0 and 5 mM K⁺ conditions. To further study the possibility of 0 mM K⁺ conditions increasing protein synthesis by enhancing translation, hERG-HEK cells pre-cultured in 5 or 0 mM K⁺ conditions for 12 h were treated with the drug cycloheximide (10 µg/ml) during the 6 h recovery period in 5 mM K⁺ culture, to prevent ribosomal activity in order to inhibit protein translation (Guo et al., 2011).
Figure 7. Tail current recovery of $I_{hERG}$ is enhanced following 0 mM $K^+$ induced internalization: effects of 0 or 5 mM $K^+$ MEM pre-culture for 12 h on $I_{hERG}$ recovery in 5 mM $K^+$ following PK digestion. After PK treatment (200 µg/ml), cells were cultured in 5 mM $K^+$ MEM at different time points and $I_{hERG}$ was recorded from 9-12 cells and summarized. Representative hERG current traces after 4-h recovery are shown above summarized time course analysis.
Figure 8. hERG (155 kDa) expression recovery is enhanced following 0 mM K⁺ induced internalization: effects of 6-h recovery after PK treatment in cells pre-cultured in 5 or 0 mM K⁺ MEM for 12 h. Lower Western blot image illustrates biotin-isolated cell-surface protein. The relative band intensities (intensity – Rel) of the 155 kDa hERG protein recovery from 0 compared to 5 mM K⁺ MEM culture are summarized below the representative Western blot image. (n=4) **, $P < 0.01$ versus 5 K⁺.
Figure 9. *hERG* mRNA expression is unaffected by 0 mM *K*⁺ MEM: effects of 0 or 5 mM *K*⁺ MEM on *hERG* transcription. The relative band intensities (intensity – Rel) of amplified *hERG* primer products from cells cultured in 0 compared to 5 mM *K*⁺ MEM for 12 h are summarized below the representative RT-PCR image (n=3).
Figure 10 and 11 shows that in the presence of cycloheximide, 0 mM K⁺ pre-culture continued to demonstrate increased recovery in IₘERG and the 155-kDa mature band expression, indicating that culture in 0 mM K⁺ pre-culture does not increase hERG recovery by enhancing hERG protein translation.

Internalized hERG channels can recycle back to the plasma membrane

The data so far suggest that the increased rate of recovery following 0 mM K⁺ culture is not due to increased hERG synthesis, we continued to study whether this phenomenon was due to hERG recycling. To determine whether internalized channels can recycle back to the plasma membrane, immunofluorescent images were obtained to study the localization of labeled hERG channels under different conditions. Cells were cultured on glass bottom plates overnight. Following culture, cell-surface HA-tagged hERG channels were labeled with anti-HA-FITC antibody at 0 h. Excess antibodies were removed with PBS washes. At 0 h, Figure 12 shows staining of membrane hERG protein. Cells were then cultured under 0 mM K⁺ MEM for 4 h. Following 4 hours of 0 mM K⁺ MEM culture, images of punctate staining were observed inside cells. Cells were then restored to 5 mM K⁺ MEM for 4 h. After restoration of cell culture in 5 mM K⁺ MEM, significant portions of internalized channels were recycled back to the plasma membrane. These data illustrate that internalized hERG channels undergo active recycling (Figure 12).

Glycan-modification occurs during internalization and retrieval trafficking of hERG channels

Our previous data showed that hERG channels are internalized under 0 mM K⁺ conditions and upon re-culturing hERG-HEK cells in 5 mM K⁺ conditions, the recovery
Figure 10. Enhanced $I_{\text{hERG}}$ recovery is unaffected by blockade of protein synthesis:
effects of cycloheximide (10 µg/ml) on 6 h $I_{\text{hERG}}$ recovery following 0 or 5 mM K$^+$ MEM
pre-culture for 12 h. Representative hERG current traces with summarized peak tail
current amplitudes are shown. The numbers in parentheses above each bar indicate the
number of cells tested from three independent trials. **, $P < 0.01$ versus 5 K$^+$. 
**Figure 11.** Enhanced hERG recovery is unaffected by blockade of protein synthesis: effects of cycloheximide on 6 h hERG recovery following 0 or 5 mM K⁺ MEM pre-culture for 12 h. The relative band intensities (intensity – Rel) of hERG protein cultured in 0 compared to 5 mM K⁺ MEM for 12 h are summarized below the representative Western blot image. (n=3) **, $P < 0.01$ versus 5 K⁺.
**Figure 12.** *Internalized hERG channels are recycled back to the plasma membrane.* Cell-surface HA-tagged hERG channels are labeled with anti-HA-FITC antibody and tracked after 4-h 0 mM K⁺ culture and back to 5 mM K⁺ for an additional 4 h. Differential interference contrast (DIC) images of cells are shown in the bottom panels.
rate of these channels is increased. Our data indicate that 0 mM K⁺ conditions do not affect protein synthesis and that internalized hERG undergo active recycling. Peculiarly, on Western blot analysis, 0 mM K⁺ culture results in the disappearance of the mature full-glycosylated 155-kDa hERG band and an increase in the immature core-glycosylated 135-kDa hERG band. Taken together, it is hypothesized that following the internalization of the 155-kDa hERG band, the mature channel is glycan-trimmed causing it to revert back to its former 135 kDa molecular weight. In this situation, the 135-kDa band would represent both the immature core-glycosylated and the maturely folded conformation. It has been previously shown that mature and immature hERG channels display different sensitivities, where the former is more resistant than the latter to digestion due to the latter’s lack of a complex folding structure to shield certain vulnerable digestion sites. To determine whether the 135-kDa band in 0 mM K⁺ cultured cells contain some glycan-trimmed mature channels, we compared the sensitivity of the 135-kDa protein to trypsin in cells cultured in 5 or 0 mM K⁺ MEM for 12 hours. Following culture, whole-cell lysates were extracted from each group and given a trypsin treatment for 5 minutes. Figure 13 shows that in comparison to 5 mM K⁺ cultured cells, the 135-kDa hERG protein in 0 mM K⁺ cultured cells displayed greater resistance to trypsin digestion.

To obtain direct evidence that the 135-kDa protein is a product of processed internalized 155-kDa hERG channels, an endocytosis assay was conducted using biotin to track the fate of internalized mature hERG protein. Because Sulfo-NHS-SS-Biotin forms a covalent linkage with primary amines, 0 mM K⁺ Tyrode solution was used alternatively to culture cells during the labeling period to avoid free amino acids found in MEM from interfering with biotin’s activity. hERG-HEK cells were cultured overnight in 5 or 0 mM
**Figure 13.** After internalization induced by 0 mM K+, hERG is glycan-trimmed. *Left panel:* The effects of trypsin digestion on hERG cultured in 0 or 5 mM K⁺ MEM for 12h. The lower (135 kDa) hERG band is more resistant to trypsin digestion at 0 mM K⁺. *Right panel:* Internalized biotin-labeled surface hERG from 0 or 5 mM K⁺ MEM pre-culture. Internalized biotinylated hERG primarily displayed a 135-kDa band. Na⁺/K⁺-ATPase was used as the plasma membrane loading control.
K⁺ MEM and then re-cultured in 0 mM K⁺ Tyrode solution with biotin (0.25 mg/ml) for 2 h, followed by a 4 h culture in 0 mM K⁺ MEM. Whole-cell lysate was collected and biotinylated protein isolated using NeutrAvidin precipitation. To ensure only internalized hERG protein was isolated, 0 mM K⁺ pre-culture was used as a negative control to demonstrate no detectable biotin-labeled channels. Figure 13 shows that following isolation of 0 mM K⁺ induced internalization of biotin-labeled hERG channels pre-cultured in 5 mM K⁺ conditions, significant portions of the hERG distribution was of the 135 kDa molecular weight. These data suggest that after hERG internalization occurs, the mature 155 kDa band is reduced to a 135 kDa band, which is then re-glycosylated to become the 155-kDa during hERG recovery.

**Rab11 interacts with hERG**

After demonstrating that hERG channels are regulated by a recycling mechanism, we continued to investigate which protein molecule(s) is involved in the regulation of hERG recycling. Previous studies have shown that Rab GTPases are important for the trafficking of ion channels; in particular, Rab4 and Rab11 participate in the recycling of various membrane proteins (Stenmark & Olkkonen, 2001). To determine whether Rab4 or Rab11 participate in the recycling of hERG channels, co-immunoprecipitation experiments were performed to determine any physical association between Rab4 or Rab11 and hERG. hERG-HEK cells were overexpressed with Rab4-GFP or Rab11-GFP. Twenty-four h after transfection, whole-cell protein was extracted from cells and immunoprecipitated with anti-GFP or anti-GAPDH antibodies. GAPDH was used as a loading negative control. Immunoprecipitated proteins were purified with washes from A/G beads, and immunoblotted to detect associated hERG expression. Figure 14 displays...
Figure 14. hERG interacts with Rab11, but not Rab4. Left panel: Whole-cell lysates from hERG-HEK cells transfected with GFP-tagged Rab4 were immunoprecipitated with anti-GFP antibody, and detected with anti-hERG antibody. Right panel: Whole-cell lysates from hERG-HEK cells transfected with GFP-tagged Rab11 were immunoprecipitated with anti-GFP antibody, and detected with anti-hERG antibody.
no interaction between Rab4 and hERG, but reveals physical association between Rab11 and hERG, displaying the presence of the 155 and 135-kDa hERG bands. Since no interaction between Rab4 and hERG was observed, the localization of endogenous Rab11 and hERG was investigated using immunofluorescence imaging. hERG-HEK cells were cultured on glass coverslips overnight. Cultured hERG-HEK cells were then cultured in 5 or 0 mM K⁺ MEM for 3 h. After culture, cells were fixed and permeabilized. Anti-Rab11 primary antibody was used to label endogenous Rab11 and anti-C20 primary antibody was used to label hERG. Immunofluorescent images from Figure 15 provide further evidence that Rab11 (red) and hERG (green) are co-localized. While hERG and Rab11 are primarily localized at the peripheries of the cell in 5 mM K⁺ MEM, the co-localization between hERG and Rab11 in 0 mM K⁺ MEM after 3 h is internalized. DIC images were required to show location and morphology of hERG-HEK cells.

Rab11 plays a role in the recovery of the hERG current from 0 mM K⁺

Upon observing that over expressed GFP-tagged Rab11 appears to be physically associated with hERG, we were next interested in testing whether Rab11 plays a role in the retrieval trafficking of internalized hERG channels. To study whether Rab11 plays a role in hERG recovery, the dominant-negative mutants of Rab4, N121I-Rab4, or Rab11, S25N-Rab11, were overexpressed in cells to disrupt their endogenous function. Cells were transiently transfected with GFP, N121I-Rab4-GFP or S25N-Rab11-GFP for 24 h. After transfection, cells were pre-cultured in 0 mM K⁺ MEM for 12 h, given subsequent PK treatment, and then re-cultured in 5 mM K⁺ MEM for 6 h. Figure 16 shows disrupting Rab11, but not Rab4, significantly delayed $I_{\text{hERG}}$ recovery from 0 mM K⁺ MEM. This result suggests that $I_{\text{hERG}}$ recovery requires Rab11 function.
Figure 15. *hERG* is co-localized with *Rab11*. Confocal images show that hERG and endogenous Rab11 are co-localized. The hERG protein was stained in green with Alexa Fluor 488 antibody. The Rab11 protein was stained in red with Alexa Fluor 594 antibody. Differential interference contrast (DIC) images of cells are shown in the left panels.
**Figure 16.** Rab11 is involved in the recovery of the hERG current. Representative currents in cells transfected with GFP, GFP tagged N121I-Rab4, or GFP tagged S25N-Rab11, recovering in 5 mM K⁺ MEM for 6 h from a 12 h pre-culture in 0 mM K⁺ are shown along with summarized peak tail current amplitudes. The numbers in parentheses above each bar indicate the number of cells tested from three independent trials. **, P<0.01 versus GFP 5 K⁺ after 0 K⁺.
Rab11 plays a role in the recovery of the mature hERG expression from 0 mM K^+.

Figure 17 displays a Western blot revealing the effects of interfering with endogenous Rab4 or Rab11 on hERG channel recovery. Cells were transfected with empty pcDNA3 vector, N121I-Rab4-GFP, or S25N-Rab11-GFP for 24 h. Following transfection, cells were pre-cultured in 0 mM K^+ MEM for 12 h, given subsequent PK treatment, and restored back to 5 mM K^+ MEM. Western blot analysis of hERG revealed that disrupting endogenous Rab11 function, but not Rab4, reduced hERG 155-kDa expression. Taken together with the last result, it suggests that Rab11 mediates hERG recycling.

Recycling of hERG channels occurs under endogenous conditions

Up to now, the data show that hERG channels appear to be recycled back to the plasma membrane upon recovery from 0 mM K^+ incubation. However, we were interested in whether hERG channels are recycled under endogenous conditions. To determine whether hERG channels are recycled under endogenous conditions, the recycling assay using two different tagged antibodies was conducted. In determining whether the hERG channel is regulated by recycling under normal conditions, it is important to differentiate recycled channels from non-recycled newly synthesized channels. hERG-HAex-HEK cells seeded onto coverslip glass were first labeled with primary anti-HA antibody for 30 min at room temperature. Following labeling, excess antibody was removed with PBS washes. Cells were then cultured at 37°C for 2 h to allow internalization of labeled cell-surface hERG channels. Un-internalized primary antibody-labeled channels are then saturated with Alexa Fluor 594 (red)-conjugated secondary antibody. The use of this antibody is to distinguish un-internalized channels.
**Figure 17.** Rab11 is involved in the retrieval trafficking of the hERG protein: effects of pcDNA3, GFP tagged N121I-Rab4, or GFP tagged S25N-Rab11 on the expression level of hERG channel proteins recovering in 5 mM K⁺ MEM for 6 h from a 12 h pre-culture in 0 mM K⁺. The relative band intensities (intensity – Rel) of the mature hERG (155 kDa) protein in the presence of GFP tagged N121I-Rab4 or GFP tagged S25N-Rab11 compared with those from pcDNA3-transfected cells are summarized beside the representative Western blot image. (n=5) **, P < 0.01 versus pcDNA3.
that remain on the cell surface, from recycled channels. After washing excess Alexa Fluor 594 (red)-conjugated secondary antibody, cells were re-incubated for an additional 2 h to allow for the recycling of previously HA-labeled internalized hERG channels back to the plasma membrane. After re-incubation, cells were treated with Alexa Fluor 488 (green)-conjugated secondary antibody, which could only bind to channels with unsaturated HA-labeled primary antibody. Figure 18 shows that recycling of internalized hERG channels occurs under normal culture conditions to regulate the trafficking of these channels back to the plasma membrane.

Rab11 is involved in the trafficking of hERG channels under normal conditions

After concluding that recycling of hERG channels likely occurs endogenously, we were interested in investigating how much recycling contributes to the maintenance of hERG density on the plasma membrane. To determine this, we examined the function and expression of hERG channels on the plasma membrane following the disruption of endogenous Rab11 by transfecting Rab11 dominant-negative mutant. Cells were transfected with empty pcDNA3 vector, N121I-Rab4-GFP, or S25N-Rab11-GFP for 24 h. Figures 19 and 20 show that disruption of Rab11, but not Rab4, decreased $I_{\text{hERG}}$ and the 155-kDa hERG expression level, respectively.

Rab11-dependent recycling of hERG traffics through the trans-Golgi network

Our data suggest that hERG channels are glycan-trimmed following internalization, and once recycled back to the plasma membrane, these channels are restored to their fully-glycosylated form. As complex modification to the core-glycan occurs in the Golgi body, it is likely that recycled channels must traffic through this organelle prior to trafficking back to the plasma membrane. To examine this hypothesis,
Figure 18. hERG Recycling occurs endogenously. Confocal images show that after 2 h, internalized hERG channels are recycled back to the plasma membrane. Anti-HA primary antibody labeled uninternalized HA-tagged hERG channels were stained in red with Alexa Fluor 594 antibody. Anti-HA primary antibody labeled recycled HA-tagged hERG channels were stained in green with Alexa Fluor 488 antibody. DIC images of cells are shown in the left panels.
Figure 19. Disruption of Rab11 under normal conditions reduces the hERG current.

Representative currents in cells transfected with GFP, GFP tagged N121I-Rab4, or GFP-tagged S25N-Rab11 are shown along with summarized peak tail current amplitudes. The numbers in parentheses above each bar indicate the number of cells tested from three independent trials. **, $P < 0.01$ versus GFP.
**Figure 20.** Rab11 is involved in the trafficking of hERG under normal conditions: effects of GFP tagged N121I-Rab4, or GFP tagged S25N-Rab11 transfection on the hERG expression level. The relative band intensities (Intensity – Rel) from cells transfected with GFP tagged N121I-Rab4 or GFP tagged S25N-Rab11 are normalized to those from cells transfected with pcDNA3 and summarized below the representative Western blot image. (n=3) **, $P < 0.01$ versus pcDNA3.
we used the drug Brefeldin A (BFA, 10 µM) to interfere with protein trafficking through the trans-Golgi network (Cheng & Filardo, 2012). hERG-HEK cells were cultured with 5 mM K⁺ MEM in the presence of BFA following pre-culture in 5 or 0 mM K⁺ conditions after Proteinase K digestion. Figures 21 and 22 show that treatment of cells with BFA (10 µM) completely blocked hERG maturation through the trans-Golgi network, as the recovery of both $I_{\text{hERG}}$ and hERG expression in the plasma membrane (155-kDa band) was blocked. From this data, it was concluded that trafficking through the trans-Golgi network is necessary for the recovery of hERG channels of newly synthesized and recycled channels to the plasma membrane.
**Figure 21.** Enhanced $I_{\text{hERG}}$ recovery is reduced by disrupting the trans-Golgi network:
effects of Brefeldin A on 6 h $I_{\text{hERG}}$ recovery in 5 mM K$^+$ MEM following 0 or 5 mM K$^+$
MEM pre-culture for 12 h. Representative hERG current traces with summarized peak
tail current amplitudes are shown. The numbers in parentheses above each bar indicate
the number of cells tested from three independent trials. **, $P < 0.01$ versus 5 K$^+$
recovery.
**Figure 22.** Disrupting the trans-Golgi network blocks retrieval trafficking of hERG channels: effects of Brefeldin A on 6 h recovery in 5 mM K+ MEM of the hERG expression level following 0 or 5 mM K+ MEM pre-culture for 12 h. The relative band intensities (Intensity – Rel) of the mature (155 kDa) hERG band from cells in each condition was normalized to the value from the recovery in the absence of BFA (10 µM) in cells pre-cultured in 5 mM K+ MEM (n=4). ***, P < 0.01 versus 5 K+ recovery.
CHAPTER 4: DISCUSSION

The density of protein in the plasma membrane is an essential determinant of hERG function, as it is crucial for the repolarization of the cardiac action potential (Sanguinetti & Tristani-Firouzi, 2006). Mutations and drugs that interfere with the normal trafficking of functional hERG channels to the plasma membrane may reduce the normal homeostatic density of the channels and cause LQTS (Keating & Sanguinetti, 2001; Sanguinetti & Tristani-Firouzi, 2006). While it is obvious that the density of hERG channels in the plasma membrane is controlled by both channel synthesis and degradation, whether the hERG channel is regulated by retrieval trafficking of internalized channels in order to maintain hERG homeostasis is not clear. Our data in the present study is the first to identify that the functional expression of cell-surface hERG channels is regulated by a recycling mechanism. Using immunocytochemical methods, we visualized the retrieval trafficking of internalized hERG channels in cells exposed to low K⁺ culture conditions, as well as under normal conditions.

Ion channel trafficking is regulated by a number of small GTPases such as Rab proteins for every step (Zerial & McBride, 2001; Grosshans et al., 2006; Pochynyuk et al., 2007; Hutagalung & Novick, 2011). To date, over 60 Rab proteins have been identified to regulate the trafficking of various proteins within cells (Bock et al., 2001). Specific to recycling, Rab4 and Rab11 are identified to mediate fast recycling from early endosomes, and slow recycling from recycling endosomes to the plasma membrane, respectively (Daro et al., 1996; Mohrmann & van der, 1999; Ullrich et al., 1996). Upon identifying that hERG channels are regulated by a recycling mechanism, we extended our focus on the roles of Rab4 and Rab11 in this pathway. Previously, our lab showed that
Rab4 indirectly regulated hERG membrane expression, as overexpression of Rab4 decreased hERG by enhancing the ubiquitin ligase of Nedd4-2 to upregulate hERG degradation (Cui & Zhang, 2013). Data in the present study showed that Rab4 is not involved in the direct mediation of hERG recycling as, co-IP analysis show no association between Rab4 and hERG, and disrupting endogenous function of Rab4 by overexpressing the Rab4 dominant-negative mutant did not affect the retrieval trafficking of internalized hERG channels. However, when the same assays were conducted to study Rab11, we found that Rab11 is associated with hERG, and overwhelming endogenous Rab11 significantly reduced the retrieval trafficking of internalized hERG channels.

To test the hypothesis that hERG channels are regulated by a recycling mechanism, we first conducted an assay to compare the recovery rate of $I_{\text{hERG}}$ in hERG-HEK cells following 0 mM K$^+$ MEM to induce hERG internalization, versus cells treated with Proteinase K, a serine protease, to clear cell-surface hERG channels. To further support the notion that this phenomenon was due to recycling of internalized hERG channels and not enhanced synthesis as a result of 0 mM K$^+$ MEM culture, hERG mRNA was analyzed and found to be unaffected by 0 mM K$^+$ MEM. As well, hERG recovery from 0 mM K$^+$ MEM was still enhanced and unaffected in the presence of cycloheximide, a drug that blocks ribosomal activity to disrupt protein synthesis (Guo et al., 2011). These data were further supported by our immunofluorescence microscopy images that displayed retrieval trafficking following recovery from 0 mM K$^+$ MEM culture. Peculiarly, under 0 mM K$^+$ MEM culture, Western blot data show a complete elimination of the 155-kDa hERG protein and an increase in the 135-kDa hERG protein. We hypothesize that the source of recyclable channels are from the 135-kDa pool and
that during the process of internalization, the mature 155-kDa hERG protein is converted into the 135-kDa hERG protein through glycan-trimming and stored within the recycling endosomes. Following 0 mM K⁺ MEM culture, the 135-kDa hERG proteins are likely composed of two separate populations of the immature newly synthesized hERG proteins and the internalized glycan-trimmed mature hERG proteins. Using a trypsin digestion assay, we found that the 135-kDa hERG protein from cells treated in 0 mM K⁺ MEM displayed greater resistance to the trypsin digestion than protein from cells treated in 5 mM K⁺. This provides further support that under 0 mM K⁺ MEM conditions, the 155-kDa hERG protein is converted into the 135-kDa hERG protein since it may maintain the mature folding conformation, which shields certain trypsin digestion sites, causing it to be less sensitive than the 135-kDa hERG protein treated in 5 mM K⁺ culture. In addition, using an endocytosis assay with biotin, to track the fate of internalized mature hERG protein, we found that biotinylated hERG proteins reverted back to the 135-kDa in 6 h under 0 mM K⁺ MEM culture. Taken together, the data suggest that internalized hERG channels are glycan-trimmed and then either targeted for recycling back to the plasma membrane or degraded through the multivesicular body/lysosomal pathway (Guo et al., 2009; Sun et al., 2011).

Our study further shows that internalized hERG channels likely traffic from endosomes to the plasma membrane through the trans-Golgi network where further complex glycosylation occurs during recycling. This notion is supported by the fact that recycled hERG channels become fully-glycosylated prior to resurfacing to the plasma membrane. As well, Rab11 is involved in hERG retrieval trafficking, and it has been shown that Rab11 docks at the trans-Golgi network in addition to residing in the
recycling endosomes. As well, BFA has been shown to disrupt the trans-Golgi network and our data show that BFA completely blocked retrieval trafficking of the internalized hERG channels (Cheng & Filardo, 2012).

In summary, the present study identifies a novel recycling pathway that regulates hERG channel expression on the plasma membrane for the first time. The hERG recycling mechanism involves Rab11-interaction, glycan-trimming during internalization, and re-extension of the glycan chain at the trans-Golgi network before being recycled back to the cell surface (Figure 23). Although 0 mM K+ MEM was used as a means to amplify the recycling process for our study, we found that retrieval trafficking occurs endogenously. The fact that interference of endogenous Rab11 function resulted in reduced hERG expression in the plasma membrane suggests that recycling plays an important role in the maintenance of homeostatic hERG expression levels in the plasma membrane. As our study has demonstrated, recycling of hERG channels may be necessary for maintaining the homeostasis of surface membrane hERG protein density. It is possible that this pathway exists to regulate hERG protein in order to conserve energy, since the channel’s structure which is unique from other VGK channels makes it more vulnerable to internalization by ligand binding. The implications for these findings are that recycling may be important for regulating the I_{Kr} in the heart. Drugs and mutations that disrupt the recycling pathway that maintain the normal homeostasis of hERG channels in ventricular cardiac tissues may cause LQTS. The limitation of this study is that the use of an immortalized cell-line to demonstrate recycling of hERG channels may not reflect the same in cardiomyocytes. As well, the global effect by the overexpression of the a dominant negative mutant form of Rab4 and Rab11 to interfere with endogenous
**Figure 23.** A diagram illustrating the hERG recycling pathway. Internalized hERG channels are initially internalized into early endosomes and sorted for recycling or degradation via the recycling endosome or late endosome, respectively. Interaction with Rab11 preferentially targets hERG channels to the recycling endosome where they are glycan-trimmed and stored. Rab11 mediates the trafficking of stored hERG channels to the trans-Golgi network for full glycosylation and then transport to the plasma membrane.
Rab4 and Rab11 may cause other effects that may not specifically reduce hERG expression.

**Future Directions**

While the recycling pathway that regulates hERG channels has been identified, the signals that mediate recycling have yet to be elucidated. In this study, we showed that certain conditions such as hypokalemia can induce vigorous internalization to promote storage of a reservoir of hERG channels. Upon restoring it to normal conditions, this reservoir of hERG channels may recycle back to the plasma membrane. This suggests that there is a feedback mechanism that is present to limit recycling. Further experiments will be conducted to understand how potassium acts as a signal to regulate this pathway.

N-linked glycosylation of hERG channels begins in the endoplasmic reticulum and further extension of the chain occurs during channel maturation in the Golgi apparatus. Disruption of the N-linked glycosylation of hERG channels at position N598 reduces hERG current and accelerates hERG turnover rate (Gong *et al.*, 2002). While N-linked glycosylation is believed to stabilize the hERG protein in the plasma membrane, the molecular mechanism that accounts for this stability is not clear. Our data show that cell-surface mature hERG channels are glycan-trimmed following internalization, and then fully-glycosylated when returning to the plasma membrane. Our data raises the possibility that N-linked glycosylation at N598 represents a signal for the sorting of internalized hERG channels, where glycosylated hERG proteins are preferentially targeted to the recycling endosomes and un-glycosylated channels are favourably targeted for the degradation pathway. It is of interest to study the role of glycosylation on hERG
trafficking as our data suggests that glycosylation may act as a signal for preferential recycling.


