

Regulation of the Human Ether-a-go-go Related Gene Potassium Channel by Neural Precursor
Cell Expressed Developmentally Down-regulated Protein 4-2 Interacting Proteins

by

Yudi Kang

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Abstract

Dysfunction of the *human ether-a-go-go related gene (hERG)*-encoded rapidly activating delayed rectifier K⁺ channel is a major cause of long QT syndrome (LQTS) due to its critical role in the repolarization of cardiac action potentials. The density of hERG channels on the cell surface, as a key determinant of its regular function, is balanced by channel trafficking to and internalization from the plasma membrane. We have shown that the E3 ubiquitin (Ub) ligase, Nedd4-2 (neural precursor cell expressed developmentally down-regulated protein 4-2), regulates hERG channel degradation by targeting the PY motif in the C-terminus of hERG channels. Interestingly, although a PY motif exists in both the immature (intracellular) and mature (cell-surface) channels, Nedd4-2 selectively degrades the mature hERG proteins. Moreover, Nedd4-2 is modulated by various proteins, such as protein kinase C (PKC). In this work, I investigated the hypotheses that the selective degradation of the 155-kDa hERG channel by Nedd4-2 is achieved by additional Nedd4 family interacting proteins (Ndfips) and that PKC signalling regulates hERG expression and function through Nedd4-2.

Using whole-cell patch-clamp, Western blot, and immunocytochemistry, I demonstrated that Nedd4-2 is directed to specific cellular compartments by Ndfip1 and Ndfip2. Ndfip1 is primarily localized in the Golgi apparatus where it recruits Nedd4-2 to target mature hERG proteins for degradation during channel trafficking to the plasma membrane. Ndfip2 mainly recruits Nedd4-2 to the multivesicular bodies (MVBs), which may impair MVBs function and impede the degradation of internalized hERG proteins. On the other hand, PKA and PKC activations increase hERG proteins on the plasma membrane by distinct mechanisms. While it is possible that PKA enhances hERG protein synthesis, PKC attenuates hERG channel degradation by inactivating Nedd4-2 via phosphorylation.

These findings extend our understanding of hERG channel regulation by Nedd4-2 and provide information useful for rescuing impaired hERG function in LQTS.

Co-authorship

Mr. Jun Guo and Mr. Wentao Li performed additional experiments used in patch-clamp data acquisition in Figure 4A and C, Figure 7, Figure 12, Figure 14, and Figure 16, especially those involving neonatal rat ventricular myocytes in Figure 12. Ms. Tonghua Yang obtained additional confocal images in Figure 9 and Figure 10.

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List of Abbreviations

BSA	Bovine Serum Albumin
Ca ²⁺	Calcium Ion
cAMP	Cyclic Adenosine Monophosphate
Cav	Caveolin
CE	Chelerythrine
CHMP3	Charged Multivesicular Body Protein 3
CHX	Cycloheximide
Cs ⁺	Cesium Ion
DTT	Dithiothreitol
ECG	Electrocardiography
ECL	Enhanced Chemiluminescent
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethyleneglycoltetraacetic Acid
ENaC	Epithelial Sodium Channel
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
FSK	Forskolin
GFP	Green Fluorescent Protein
HECT	Homologous to E6-AP-COOH Terminus
HEK	Human Embryonic Kidney
hERG	human Ether-à-go-go Related Gene
HRP	Horseradish Peroxidase

IBMX	3-isobutyl-1-methylxanthine
I_{K1}	Inward Rectifier Current
I_{CaL}	Long-lasting Calcium Current
I_{hERG}	hERG Current
I_{KACh}	Acetylcholine-activated Inward-rectifying Potassium Current
I_{Kr}	Rapidly Activating Delayed Rectifier Potassium Current
I_{Ks}	Slowly Activating Delayed Rectifier Potassium Current
I_{Kur}	Ultra Rapidly Activating Delayed Rectifier Potassium Current
I_{Na}	Sodium Current
I_{to}	Transient Outward Current
K^+	Potassium Ion
LQTS	Long QT Syndrome
MEM	Minimum Essential Medium
MinK	Minal Potassium Subunits
MiRP	MinK-related Peptide
MVBs	Multivesicular Bodies
N4WBP5	Nedd4 WW Domain Binding Protein 5
Na^+	Sodium Ion
Ndfip	Nedd4 Family Interacting Protein
Nedd4	Neural Precursor Cell-expressed Developmentally Downregulated protein 4
PBS	Phosphate- Buffered Saline
PK	Proteinase K
PKA	Protein Kinase A

PKC	Protein Kinase C
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethanesulfonyl Fluoride
PVDF	Polyvinylidene Difluoride
RING	Really Interesting New Gene RIPA Radioimmunoprecipitation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Electrophoresis
SGK	Serum/Glucocorticoid regulated Kinase
siRNA	small interfering RNA
SQTS	Short QT Syndrome
TBS	Tris-Buffered Saline
Ub	Ubiquitin
WT	Wild Type

Chapter 1: Introduction and Literature Review

The *human ether-a-go-go related gene (hERG)*-encoded K^+ channels play an essential role in cardiac repolarization. Due to their unique gating properties, they are critical in preventing early afterdepolarization (EAD), a common risk factor for cardiac arrhythmia. The dysfunction of hERG channels as a result of gene mutation or drug blockade can lead to long QT syndrome (LQTS), which put affected individuals at risks of syncope, arrhythmia, and sudden cardiac death.

Given the critical role of hERG channel function, which depends highly on its expression on the cell surface, the balance between hERG protein synthesis and subsequent trafficking to the plasma membrane and degradation of surface-expressed hERG channels must be closely monitored. The surface expression of hERG can be affected by multiple factors, including the temperature, serum K^+ level, and as well as stress and excitement. While most *hERG* mutations result in impaired forward trafficking, the degradation of wild type (WT) hERG channels has been shown to be regulated by the E3 ubiquitin ligase Nedd4-2. Nedd4-2 is further mediated by several molecules and cell signalling pathways including the small GTPase Rab4, serum glucocorticoids activated kinases (SGK1 and SGK3), and protein kinase C (PKC). It has also been shown that two Nedd4 family interacting proteins (Ndfip1 and Ndfip2) may play roles in Nedd4-2 activation and translocation.

Activation of adrenergic receptors, which leads to activation of PKA and PKC, has been implicated in abnormal cardiac events, including cardiac arrhythmia. However, there is no consensus in current literature on whether PKA or PKC activation increases or decreases I_{Kr} /hERG channel function. In fact, the inactivation of Nedd4-2 by PKC-mediated phosphorylation may be involved.

1. Cardiac Repolarization and LQTS

The ventricular action potential (AP) is comprised of various ion influx and efflux in cardiomyocytes, and is shown in Figure 1. While cardiac depolarization is triggered by the large and rapid influx of Na^+ ions, its repolarization, as a result of K^+ efflux, is essential to bring the membrane potential back to the resting state so that the subsequent AP can take place.

The cardiac Na^+ current (I_{Na}) is mainly conducted by the voltage gated Na^+ channel $\text{Nav}1.5$, which has its pore-forming α -subunit encoded by *SCN5A* (Abriel, 2010). While the loss-of-function mutations in *SCN5A* are associated with Brugada Syndrome, the gain-of-function mutations, resulting in elevated inward Na^+ current, are linked with the congenital long QT syndrome type 3 (LQTS3) (Ruan *et al.*, 2009).

The outward K^+ current contributing to repolarization is conducted by several K^+ channels, each with specific characteristics (Grant, 2009). Malfunction of any of these channels can increase or decrease the flow of outward K^+ ions, leading to shortened or prolonged QT interval on an electrocardiogram (ECG), respectively.

A shortened QT interval, or short QT syndromes (SQTS), has been associated with both atrial and ventricular arrhythmias, including atrial fibrillation, polymorphic ventricular tachycardia, and ventricular fibrillation (Giustetto *et al.*, 2006; Viskin *et al.*, 2004). SQTS is believed to be arrhythmogenic because of its heterogeneity affecting either the epicardium or the endocardium. The different degree of refractoriness between epicardium and endocardium predisposes the heart to reentry and arrhythmia.

Compared to SQTS, long QT syndrome (LQTS) is more frequent. A prolonged QT interval on an ECG is the diagnostic mark for LQTS. As the first genetically identified cardiac disorder, congenital LQTS is the most common cardiac channelopathy (Kim, 2014), predisposing affected

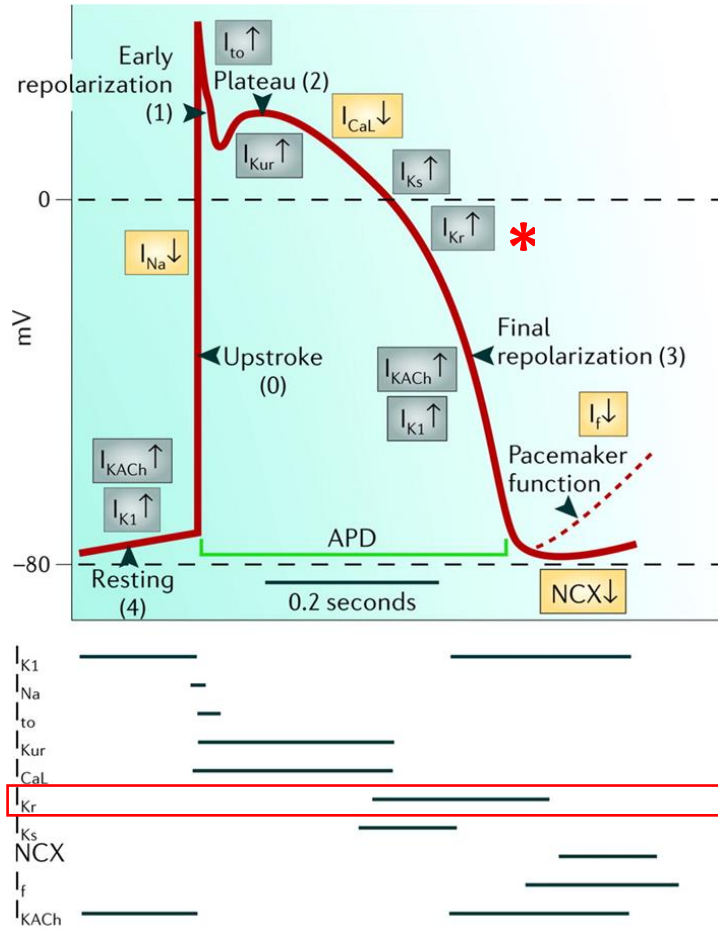


Figure 1: Ventricular action potential comprised of currents conducted by various ion channels.

Five phases constitute the ventricular action potential. Outward currents are indicated in grey boxes, and inward currents are in yellow boxes. The resting membrane potential (4) is maintained by the outward K^+ current I_{KACh} and I_{K1} . The upstroke (0) is a result of Na^+ ion influx (I_{Na}). Phase 1 (early repolarization) is caused by Na^+ channel inactivation and the outflow of K^+ current I_{to} . The following plateau (2) is reached by Ca^{2+} influx I_{CaL} and K^+ efflux I_{Kur} and I_{Ks} . The final repolarization (3) is mainly contributed by I_{Ks} , I_{Kr} , I_{KACh} and I_{K1} . The phase that each current contributes in an action potential is shown at the bottom by the lines. I_{Kr} is emphasized by red asterisk in the graph and red box underneath (image modified from Grant, 2009).

individuals to high risks of ventricular tachycardia, syncope, and sudden death. Although the heterogeneity of myocardium also underlies the arrhythmogenesis of LQTS (Antzelevitch, 2007), the reactivation of L-type Ca^{2+} channel, which results in early afterdepolarization (EAD), can eventually induce Torsades de pointes (TdP) (Lankipalli *et al.*, 2005). To date, 13 types of LQTS have been linked to mutations in genes encoding the pore-forming subunits of ion channels or their associated proteins. The most common, and thus clinically important ones, are LQT1 (30% - 35% of cases), LQT2 (25% - 30% of cases), and LQT3 (5% - 10% of cases), which are caused by the loss-of-function mutations in *KCNQ1*, *KCNH2*, and the gain-of-function mutations in *SCN5A*, respectively (Splawski *et al.*, 2000). LQTS can also be induced by factors including disease, drug blockade (Obers *et al.*, 2010) and electrolyte abnormalities such as hypocalcemia, hypokelamia and hypomagnesemia (Gordon *et al.*, 2008). Ironically, many antiarrhythmic drugs, including quinidine, block outward K^+ current, and give rise to acquired LQTS (Benz & Kohlhardt, 1994; Clark *et al.*, 1995).

2. The hERG K^+ channel

The *human ether-a-go-go related gene (hERG)* or *KCNH2* on chromosome 7 was discovered in 1994 (Warmke & Ganetzky, 1994). It encodes the pore forming α -subunit of the voltage-gated K^+ channel (Kv11.1) that conducts the rapidly activating delayed rectifier K^+ current (I_{Kr}) (Sanguinetti *et al.*, 1995; Trudeau *et al.*, 1995). I_{Kr} plays an essential role in repolarization (phase 3) of the action potential in cardiac myocytes. Similar to other voltage-gated K^+ channels, each hERG channel subunit contains six transmembrane domains ranging from S1 to S6. S1 to S4 constitute the voltage sensing complex with the S4 domain carrying six positively charged amino acids. These positive amino acids in S4 cause it to move toward the outside of the plasma membrane when the cell is depolarized, triggering the channel to open. The

S5 and S6 domains form the pathway conducting K^+ current. The amino acid sequence G-Y/F-G in the pore loop between S5 and S6 domains confers the ion selectivity of hERG channel for K^+ (Doyle *et al.*, 1998). Kv11.1 channels are homotetramers with the pore-forming domains of the α -subunits facing towards each other constituting the ion pathway (Figure 2).

To date, approximately 300 LQTS-associated mutations have been discovered in *hERG*. In 1995, mutations in *hERG* were linked to type 2 long QT syndrome (LQT2) (Curran *et al.*, 1995). Most *hERG* mutations cause misfolding of the protein subunits, leading to impaired forward trafficking of the channel from the endoplasmic reticulum (ER) to the cell membrane (Delisle *et al.*, 2004). Other mutations may alter hERG gating properties, such as enhanced channel inactivation, resulting in surface expressed but non-functional K^+ channels (Nakajima *et al.*, 1998).

Two accessory proteins have been shown to interact with hERG channel as β -subunits: MinK, encoded by *KCNE1*, and MinK-related peptide 1 (MiRP1), encoded by *KCNE2*. Mutations in both *KCNE1* and *KCNE2* have been linked with LQTS (Abbott *et al.*, 1999; Anantharam & Abbott, 2005). Although some believe that MinK is the β -subunit of KCNQ1 (I_{Ks}), MinK and MiRP1 associate differently with hERG channel in a way that MinK co-localizes better with hERG channel at the ER while MiRP1 is more abundant at the cell surface (Um & McDonald, 2007). Furthermore, there are more MiRP1 proteins in ventricles than in atria of rat and guinea pig hearts. What is also surprising is that the phosphorylation of MiRP1 accelerates hERG degradation, thus reduces its surface expression (Zhang *et al.*, 2012). In terms of electrophysiology, cells co-expressing hERG-MinK produces higher current density, through the stabilizing effect of MinK on hERG channel, compared to those expressing hERG channel alone

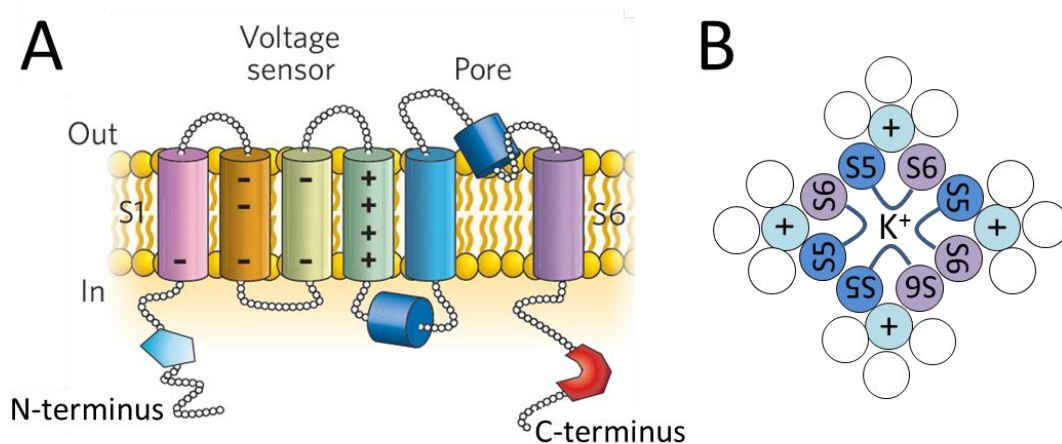


Figure 2: Schematics of hERG channel structure.

(A) Diagram of a single hERG channel subunit. Each cylinder represents one transmembrane domain (S1 to S6 from left to right). S1 to S4 constitute the voltage sensor with S4 domain carrying 6 positively charged amino acids. S5 and S6 form the pore conducting K⁺ current. The N-terminal Per-Arnt-Sim (PAS) domain (light blue pentagon), C-terminal cyclic nucleotide binding (cNDB) domain (red polygon), S4-S5, and S5-pore linkers (blue cylinders) are also indicated (modified from Sanguinetti & Tristani-Firouzi, 2006). (B) Diagram of 4 subunits of hERG forming a Kv11.1 channel. These subunits are oriented with pores facing towards each other forming the ion pathway. The positively charged S4 domain, and pore-forming S5 and S6 domains are denoted in colors corresponding to those in panel (A).

(McDonald *et al.*, 1997). On the other hand, MiRP1 decreases current density, shifts the activation curve to more positive voltages, and increases its deactivation rate when it is co-expressed with hERG channel (Abbott *et al.*, 1999). Such depressed hERG channel current in the presence of MiRP1 better resembles native I_{Kr} .

2.1 hERG Channel Gating

Similar to other voltage-gated K^+ channels, Kv11.1 channel exists in one of the three states: closed, open, and inactivated. Kv11.1 channel possesses unique gating properties. Its activation is unusually slow, while its inactivation is very fast and voltage-dependent. Such gating properties result in inward rectification: substantially larger inward current at negative voltages than outward current at positive voltages (Schönherr & Heinemann, 1996; Smith *et al.*, 1996; Spector *et al.*, 1996). The recovery of Kv11.1 channel from inactivation is fast, resulting in a unique hooked tail current upon repolarization following depolarization. This tail current is often used to assess the function of Kv11.1 channel. Its slow deactivation combined with the fast recovery from inactivation allows the channel to pass significant amount of current once it opens, which is critical for maintaining the plateau phase of action potential. This unique gating property is crucial, not only in controlling normal cardiac repolarization, but also in the suppression of premature beats (Sanguinetti & Tristani-Firouzi, 2006). The slow activation and fast voltage-dependent inactivation at depolarized membrane potentials of Kv11.1 channels allow little K^+ current to pass through the channel during phase 1 and 2 of ventricular action potential. Furthermore, if a premature beat does occur, a large outward K^+ current can antagonize the depolarizing effect, and prevent it from taking place (Lu *et al.*, 2001).

2.2 Regulation of Protein Surface Expression

Regulation of the Kv11.1 channels is essential for the homeostasis of their surface expression and the electrical conduction of cardiac myocytes. The expression of Kv11.1 channels on the cell membrane is determined by a balance between protein synthesis followed by anterograde trafficking to and the retrograde internalization of old channels from the plasma membrane. Both pathways are closely regulated by various cellular signals.

2.2.1 Biosynthesis and Forward Trafficking

The hERG mRNAs are processed in the nucleus, translated by ribosomes and assembled into tetramers in the ER. The nascent hERG polypeptide is folded with the help of chaperones presented at the ER or in the cytosol (Ficker *et al.*, 2003; Nanduri *et al.*, 2009). Upon correct folding and release of the chaperones, individual hERG proteins then undergo asparagine (N)-linked glycosylation. Misfolded proteins as a result of genetic mutation remain bound to the chaperones and are retained in the ER (Wang *et al.*, 2012). Prior to N-linked core-glycosylation, each hERG subunit has a molecular weight of 132 kDa. Although hERG proteins contain two extracellular N-linked glycosylation sites, N598 and N629, only N598 is core-glycosylated (Petrecca *et al.*, 1999). Addition of mannose results in the immature hERG protein with a molecular weight of 135 kDa on a Western blot (Gong *et al.*, 2002). While it was originally believed that N-linked glycosylation is required for hERG forward trafficking (Petrecca *et al.*, 1999), it has been shown that the unglycosylated proteins from cells treated with tunicamycin, which inhibits core-glycosylation, are also functional on the plasma membrane, albeit less stably (Gong *et al.*, 2002).

After its assembly and core glycosylation in the ER, immature hERG channel proteins (135 kDa) is transferred to the Golgi apparatus to undergo full glycosylation and thereby reach a

molecular weight of 155 kDa (Gong *et al.*, 2002). The extra 20-kDa glycan chain has been shown not only to enhance hERG channel stability on the plasma membrane (Gong *et al.*, 2002), but also to protect it from drug blockade. The K⁺ channel regulatory protein, KCR1, has been shown to reduce the sensitivity of hERG channel to drug blockade, however it is only effective when the channels are fully glycosylated (Nakajima *et al.*, 2007). Evidence also suggests the protective role of N-glycosylation against protease digestion (Muthusamy *et al.*, 2015; Hayashi & Yamashita, 2012). Those fully glycosylated mature hERG proteins are then transported to the cell membrane as functional K⁺ channels with a half-life of about 11 h (Ficker *et al.*, 2003).

2.2.2 Endocytosis and Degradation

While ion channels can be regulated by adjusting gating properties, their quantitative change on the cell surface is also essential in controlling cardiac excitability. When the channel is on the cell surface, it is regulated by several extracellular conditions. It has been proven that extracellular K⁺ concentration is a key determinant of hERG channel surface expression, and low extracellular K⁺ level significantly decreases surface-expressed hERG channels (Guo *et al.*, 2009; Sun *et al.*, 2011). Since the whole-cell current amplitude largely reflects the density of surface-expressed functional channels, the channel production and degradation must reach a balance to maintain the optimal function.

The protein endocytosis pathways can be clathrin dependent or independent (Doherty & McMahon, 2009). Clathrin is essential in coating vesicles for internalization. The clathrin-dependent pathway involves the formation of clathrin-coated pits that enclose the target protein. The pits are then internalized and released when the target protein fuses with other cellular vesicles (Sorkin & von Zastrow, 2009). hERG proteins, however, undergo clathrin-independent caveolin dependent endocytosis. The specialized lipid rafts are formed with the integral

membrane protein caveolin (Cav) molecules inserted at the cytoplasmic side of the lipid bilayer which also contains hERG proteins. There are 3 subtypes of Cav molecules, Cav-1, Cav-2 and Cav-3. While Cav-1 and Cav-2 are found in non-muscle cells, Cav-3 is present in muscle cells (Hansen & Nichols, 2009). Our lab has demonstrated that the cell surface expression of hERG channels is regulated by Cav-3 through E3 Ub ligase Nedd4-2. We found that Cav-3, hERG channel and Nedd4-2 form a complex at cell surface at the time of endocytosis. Elevated Cav-3 expression enhances the interaction between Nedd4-2 and hERG channel, which results in higher level of hERG channel ubiquitination, and therefore greater rate of degradation (Guo *et al.*, 2012).

The endocytosis of hERG proteins can be accelerated with extracellular stimuli. It is well known that hERG channels can be blocked by various drugs, including antiarrhythmic agents. However, evidence has shown that some drugs impair hERG channel function by inducing protein endocytosis. Amoxapone and desipramine, prescribed as antidepressants, have been reported to cause hERG channel internalization in hERG-HEK cells (Dennis *et al.*, 2011; Obers *et al.*, 2010). Furthermore, desipramine was found to induce hERG channel degradation by the 26S proteasome through the polyubiquitination – proteosomal pathway (Dennis *et al.*, 2011). Hypokalemia, one of the risk factors for LQTS, has also been shown to impair hERG channel function through enhanced protein degradation. Our lab has demonstrated that under 0 mM extracellular K^+ condition, the surface-expressed channels undergo conformational changes, which makes them unstable and easily internalized. The internalized proteins are degraded through monoubiquitination – lysosomal pathway via multivesicular bodies (MVBs) in hERG-HEK cells (Guo *et al.*, 2009).

3. Nedd4 family proteins and Nedd4-2

Ubiquitination is an essential posttranslational process that controls biological signalling in many cellular pathways. The Ub tag may serve as a signal for protein endocytosis, trafficking, sorting, and degradation in multiple cellular compartments. Protein ubiquitination requires the conjugation of the C-terminal carboxyl group of Gly from the Ub with the ϵ -amino group of a Lys from the target protein (Ciechanover, 2005; Hershko & Ciechanover, 1998). An Ub molecule has seven Lys binding sites. While K48-linkage targets substrate for proteosomal degradation (Ciechanover, 2005; Hershko & Ciechanover, 1998), K63-linkage directs substrates to lysosomal pathway (Hicke & Dunn, 2003). The entire process of ubiquitination involves the activation of Ub by E1 Ub-activating enzymes, the conjugation of Ub to the E2 Ub-conjugating enzymes and the cleavage followed by the transfer of Ub from E2 to substrate by the E3 Ub ligases, including Nedd4 family proteins. This three-tiered energy-dependent labeling process allows the control of protein degradation to occur at the desired level and cellular location, since one E1 Ub-activating enzyme is capable of activating the whole downstream process in most organisms including human (Pickart, 2001).

As the enzymes responsible for the final step of ubiquitination, the E3 Ub ligases regulate the specificity of the process. There are more than 600 putative E3 ligases in mammalian cells, divided into two main classes: the Really Interesting New Gene (RING) and the Homologous to E6-AP C-Terminus (HECT) (Rotin & Kumar, 2009). The Nedd4 family proteins belong to the HECT Ub ligases, and are comprised of 9 members including Nedd4, Nedd4-2 (Nedd4 like, Nedd4L), Itch, Smurf1, Smurf2, WWP1, WWP2, NedL1 and NedL2 (Ingham *et al.*, 2004; Harvey & Kumar, 1999; Shearwin-Whyatt *et al.*, 2006). Nedd4-2, the focus of the current study, is closely related to Nedd4, and both genes have been shown to be present in the genomes of all

vertebrates. They contain an N-terminal calcium/lipid and/or protein binding C2 domain, 2 to 4 WW domains containing 35-40 amino acids with two conserved Trp (W) residues for their binding with the P/LPXY motif from the substrates, and a C-terminal catalytic HECT domain as shown in Figure 3 (Ingham *et al.*, 2004).

3.1 Nedd4-2

Unlike Nedd4 homologs, which are found in all eukaryotes, Nedd4-2 is thought to have appeared later in the evolutionary process and is only found in vertebrates (Yang & Kumar, 2010). It is ubiquitously expressed, with higher amounts in liver, kidney, heart, and lung (Araki *et al.*, 2008). Nedd4-2 is best characterized in the regulation of the epithelial Na⁺ channel (ENaC), which is responsible for Na⁺ reabsorption and mediating salt and water homeostasis. Extensive *in vitro* studies have established the fact that Nedd4-2 is a critical molecule in regulating channel endocytosis and degradation through direct interaction between its WW domains and the PY motif in the C-terminus of ENaC (Fotia *et al.*, 2003; Harvey *et al.*, 1999; Staub *et al.*, 1996). The disruption of the C-terminus in ENaC that impairs its interaction with Nedd4-2 was also found to be the cause of Liddle's syndrome with symptoms of severe sodium retention and hypertension (Staub *et al.*, 2000). The *in vitro* evidence was confirmed by *in vivo* studies showing that the "Liddle's" mouse with salt-sensitive hypertension can be created by mutating the PY-motif in ENaC (Pradervand *et al.*, 2003) or by depleting endogenous Nedd4-2 (Shi *et al.*, 2008).

Nedd4-2 has also been shown to regulate other proteins. Nedd4-2 overexpression in *Xenopus* oocytes strongly suppresses the function of several voltage-gated sodium channels, which are responsible for generating and propagating action potentials (Fotia *et al.*, 2004; Rougier *et al.*, 2005; van Bemmelen *et al.*, 2004). It also regulates the degradation of serum- and

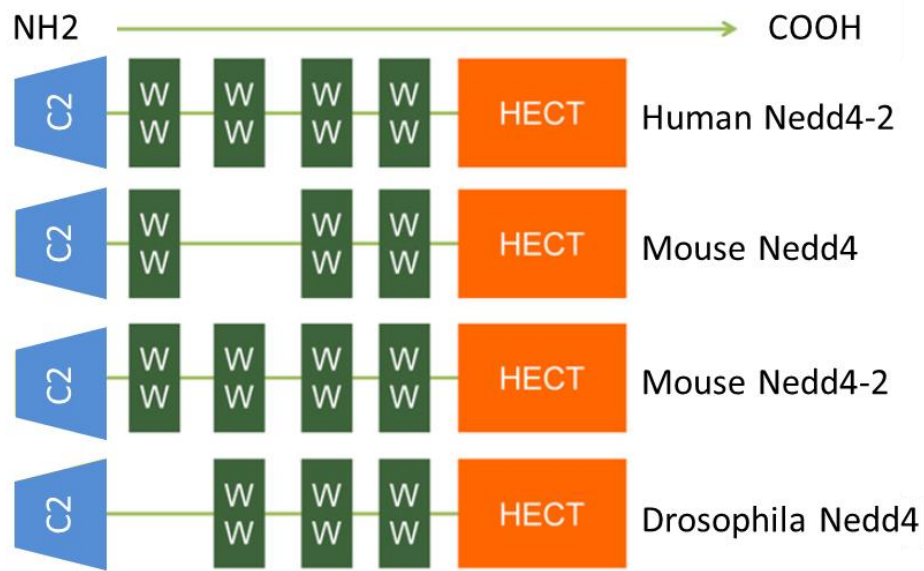


Figure 3: Schematics representing the structures of Nedd4 and Nedd4-2.

Nedd4 family proteins contain an N-terminal calcium/lipid and/or protein binding C2 domain (blue), 2 to 4 WW domains (green) for their binding with the P/LPXY motifs from the substrates, and a C-terminal catalytic HECT domain (orange). In absence of substrate, the C2 domain winds back to bind weakly to its own HECT domain, concealing its catalytic activity (modified from Donovan & Poronnik, 2013).

glucocorticoid-inducible kinase (SGK) 1 (Zhou & Snyder, 2005) and the surfactant protein C (Conkright *et al.*, 2010), in that the overexpression of Nedd4-2 decreases the expression levels of both proteins.

Given the essential role of Nedd4-2 in regulating protein levels, its optimal protein expression level and function are required.

3.1.1 Activation by Ndfips

The activation of Nedd4-2 is poorly understood. Nedd4-2 naturally exists in an autoinhibitory state, with the C2 domain binding to the HECT domain, concealing its catalytic activity. Increased intracellular Ca^{2+} has been shown to activate Nedd4-2 by disrupting the interaction between the C2 and HECT domains, and as a result, the ubiquitination of Nedd4-2 substrate ENaC is enhanced (Wang *et al.*, 2010).

In 2002, two novel proteins that interact and possibly activate Nedd4 family proteins were identified, and were named Ndfips. Ndfip1 was discovered as a Nedd4 WW domain interacting protein (N4WBP5), and Ndfip2 (N4WBP5A) was later discovered and is highly homologous to Ndfip1 (Harvey *et al.*, 2002; Konstas *et al.*, 2002; Cristillo *et al.*, 2003). Ndfip1 and Ndfip2 have three transmembrane domains and two PY motifs at the N-terminals (GeneBank, Ndfip1 ID: 80762 and Ndfip2 ID: 54602). Ndfip1 was found to be a potential target for ubiquitination by Nedd4 family proteins, including Nedd4, Nedd4-2, KIAA0332, WWP2, AIP-4 and Itch (Harvey *et al.*, 2002). The same authors also found that Ndfip1 is localized in the Golgi apparatus and its overexpression disrupts this organelle. Through its interaction with Nedd4 family proteins, Ndfip1 plays critical roles in human cortical development by increasing Nedd4-2 specificity in removing undesired substrates (Goh *et al.*, 2013), regulation of divalent metal transporter 1 (DMT1) in human neurons by increasing transporter degradation via Nedd4-2 upon divalent

metal exposure (Howitt *et al.*, 2009), and neuroprotection during ischemic stroke by assisting Nedd4-2 in removing ubiquitinated proteins (Lackovic *et al.*, 2012). Ndfip2 (N4WBP5A), localized primarily in MVBs (Shearwin-Whyatt *et al.*, 2004), was found to upregulate the surface expression of ENaC by reducing Nedd4-2-mediated degradation, since Nedd4-2 are occupied by Ndfip2 (Konstas *et al.*, 2002).

3.1.2 Inhibition by Phosphorylation

While Nedd4-2 can be activated by Ndfips, their activity can be terminated by phosphorylation. More precisely, phosphorylation impairs the binding of Nedd4-2 to its substrates. The effect of Nedd4-2 phosphorylation is better understood in the regulation of ENaC. SGK1 phosphorylates Nedd4-2 at the consensus sequences between WW domains 1 and 2 at Ser 221 and Thr 246, and domains 2 and 3 at Ser327, which then fails to target ENaC. Moreover, mutations of these three sites abolish the effects of SGK and PKA on ENaC, since mutant Nedd4-2 constitutively binds to ENaC (Chen *et al.*, 1999). The mechanism of the phosphorylation in preventing the ubiquitination of ENaC by Nedd4-2 is not clear, but evidence suggests that 14-3-3 protein binds to Nedd4-2 when it is phosphorylated, blocking its binding to substrates (Bhalla *et al.*, 2005; Ichimura *et al.*, 2005). In addition, Nedd4-2 can also be phosphorylated by PKA when the intracellular cyclic AMP (cAMP) level is increased as a result of vasopressin release in response to reduced intravascular volume (Snyder *et al.*, 2004). Nedd4-2 can be phosphorylated by PKC in response to muscarinic receptor activation in hERG-HEK cells as well (Wang *et al.*, 2014).

3.1.2.1 Protein kinase A (PKA)

PKA, also known as cAMP-dependent protein kinase or A kinase, is a serine/threonine kinase that modifies the function of target proteins by phosphorylation. cAMP is synthesized

from adenosine triphosphate (ATP) by adenylyl cyclase, and is degraded by phosphodiesterases (PDEs) (Sunahara *et al.*, 1996; Fimia & Sassone-Corsi, 2001). PKA is a tetramer with each subunit comprised of a catalytic subunit and a regulatory subunit. The catalytic site of PKA is hindered by the regulatory subunits under the inactive state. PKA is activated when its regulatory subunit is displaced upon the binding of cAMP, and when the catalytic site is exposed and phosphorylated at T197 (Johnson *et al.*, 2001; Cauthron *et al.*, 1998). There are two classes of PKA, PKA(I) and PKA(II), due to differences in the regulatory groups, which are expressed differentially in tissues (Corbin *et al.*, 1975; Taylor *et al.*, 1990).

PKA acts at discrete domains within the cell, therefore its specificity requires the assistance of other proteins such as the A kinase anchoring proteins (AKAP's). A large number of AKAP's relating to specific substrates have been identified (Bauman & Scott, 2002), and it has been suggested that AKAP directs PKA to specific cellular location to facilitate its binding with cAMP (Faux & Scott, 1996). Some of the potential targets of PKA phosphorylation include enzymes, ions channels, chromosomal proteins and transcription factors (Shabb, 2001).

3.1.2.2 Protein kinase C (PKC)

Similar to PKA, the isoforms of PKC are also serine/threonine kinases. PKC is a single peptide comprised of an N-terminal regulatory domain and a highly conserved C-terminal catalytic domain (Newton, 1995). There are more than 18 isoforms of PKC classified into three groups according to their structures and cofactor regulation: the conventional group, the novel group and the atypical group (Steinberg, 2012). The conventional PKC isoforms binds to diacylglycerol (DAG) or phorbol ester, as well as anionic phospholipid in a Ca^{2+} -dependent manner. The novel PKC isoforms, however, do not bind to Ca^{2+} . The atypical PKCs bind to PIP_3 and ceramide but not to DAG.

PKC isoforms are distributed specifically in different tissues and cell types. In the heart, PKC α (conventional) plays an important role in the regulation of cardiac contraction by sensing Ca²⁺ level (Braz *et al.*, 2004), whereas the level of PKC β (conventional) was increased in failed hearts (Bowling *et al.*, 1999). PKC δ (novel) is associated with cardiac ischemia and hypertrophy (Chen *et al.*, 2001). Furthermore, the level of atypical PKC's is elevated during cardiac hypertrophy, which then decline when the disease progresses to congestive heart failure (Koide *et al.*, 2003).

3.1.2.3 Activation of cardiac PKA or PKC pathway

The effects of PKA and PKC on the cardiovascular system and especially on the heart are exerted through the adrenergic signalling pathway. Elevated catecholamines, including epinephrine and norepinephrine, results in increased activation of the G-protein coupled adrenergic receptors (AR's), such as the α (α -AR) and β subtypes (β -AR).

The α -AR, particularly α_1 adrenoceptor, is associated with Gq proteins (Ron & Kazanietz, 1999). The activation of Gq protein stimulates phosphatidyl inositol-specific phospholipase C β (PLC β), which hydrolyzes phosphatidyl-4,5-bisphosphate (PIP₂) to the intracellular second messengers 1,4,5-inositol-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ induces the intracellular mobilization of Ca²⁺, whereas DAG activates PKC. There are three sub-types of α_1 -ARs, α_1A , α_1B , and α_1D , all of which are present in the heart despite their differential expression in tissues (Jensen *et al.*, 2011). α_1A and α_1B are the predominant types expressed in the cardiomyocytes, whereas α_1D is mainly expressed in the coronary smooth muscle cells (Jensen *et al.*, 2009b). The α_1 -ARs in the heart are critical for its post-natal growth, and serve compensatory roles in that they increase in abundance and function while those of β -ARs are

downregulated under chronic stress, such as heart failure (Jensen *et al.*, 2009a; Bristow *et al.*, 1982).

β -ARs are the more predominate type in the heart, and comprise 3 sub-types, β_1 - β_3 , with β_1 being the most abundant (Steinberg, 1999; Brodde *et al.*, 2006). β_1 and β_2 are the main mediators responding to adrenergic stimulation. Their activations can stimulate Gi or Gs, causing a decreased or an increased production of cAMP, respectively, by adjusting the activity of adenylyl cyclase (Kaumann & Molenaar, 1997). The altered cAMP level leads to the corresponding change of PKA activity.

The autonomic nervous system (ANS) including both α - and β -AR are involved in the sympathetic stimulation of heart to enhance cardiac performance by increasing heart rate, contractility, atrioventricular conduction, and reducing venous capacitance. While the short term stimulation of AR is beneficial to prepare the heart for the “fight and flight” response, chronic activation triggers pathological cardiac remodeling, hypertrophy, heart failure, and arrhythmia (Movsesian & Bristow, 2005; Morisco *et al.*, 2001; Engelhardt *et al.*, 1999; Antos *et al.*, 2001; Workman, 2010). Furthermore, as mentioned above, while adrenergic stimulation activates downstream signalling cascade of PKC and PKA, definitive effects of PKA and PKC on hERG channels are still controversial, since both enhancement and suppression of I_{K_r}/I_{hERG} have been reported. hERG protein synthesis is enhanced at the level of the ER upon PKA activation (Chen *et al.*, 2009), whereas reduced I_{K_r} (I_{hERG} in native cells) was found in cardiomyocytes of guinea-pig upon β_1 adrenergic activation via PKA pathway (Karle *et al.*, 2002). On the other hand, while increased hERG protein was observed with α_1 adrenergic activation via the PKC pathway (Chen *et al.*, 2010), the same adrenergic activation through PKA-dependent PKC pathway reduced I_{K_r} in guinea-pig hearts (Wang *et al.*, 2009). Such discrepancy raises the possibility that the effects

of PKA and PKC on hERG channels may not be direct. However, none of the above studies investigated the effects of PKA and PKC activations on hERG channels through Nedd4-2. To my knowledge, so far, only one study demonstrates that muscarinic receptor activation enhances hERG channel surface expression and function by inhibiting Nedd4-2 via PKC phosphorylation (Wang *et al.*, 2014).

4. Hypothesis and Study Objectives

As an E3 Ub ligase, Nedd4-2 regulates hERG channel degradation. However, whereas both the 155-kDa and the 135-kDa hERG proteins contain a PY motif, Nedd4-2 only targets the 155-kDa form. It is unlikely that the PY motif in the 135-kDa hERG protein is concealed whereas that in 155-kDa is not, since the 135-kDa and the 155-kDa hERG proteins are only different in their glycosylation levels. Ndfips have been shown to be present at cellular compartments where only the 155-kDa form is observed. Therefore, I hypothesize that Ndfips direct Nedd4-2 to distinct cellular compartments where only the 155-kDa forms are ubiquitinated by Nedd4-2.

To examine this hypothesis, I accomplished the following objectives:

1. Examine the effects of Ndfip1 and Ndfip2 overexpression on hERG protein expression and function.
2. Examine the change in cellular location of Nedd4-2 and hERG channels upon Ndfip1 and Ndfip2 overexpression.

As mentioned above that the effect of adrenergic activation and thus PKA and PKC activations on I_{Kr} /hERG function is still controversial, we examined the effect of PKA and PKC activations on hERG channel expression and function. We have proven that muscarinic receptor activation augments hERG channel expression and function by inhibiting Nedd4-2 via PKC-mediated phosphorylation, and since PKA is also part of the downstream signalling of adrenergic activation, I hypothesize that PKA and PKC activations can both increase hERG channel expression and function, however, possibly through distinct mechanisms.

To test this hypothesis, I achieved the following objectives:

1. To test the effect of PKA and PKC on hERG channel expression and function.
2. To test the effect of PKA and PKC on phosphorylation level of Nedd4-2.

Chapter 2: Materials and Methods

1. Molecular biology

hERG cDNA was provided by Dr. Gail Robertson (University of Wisconsin – Madison); the human embryonic kidney (HEK) 293 cell line stably expressing hERG channels (hERG-HEK cell line) was provided by Dr. Craig January (University of Wisconsin – Madison). The Myc-tagged human Ndfip1 and Ndfip2 cDNAs were provided by Dr. Thomas Mund (Medical Research Council Laboratory of Molecular Biology, Cambridge, UK). The human Nedd4-2 plasmid in pBluescript II was obtained from Kazusa DNA Research Institute (Chiba, Japan). The open reading frame was amplified using PCR and cloned into HA-pcDNA3 (Invitrogen) to generate HA-tagged Nedd4-2 (Nedd4-2-HA). The scrambled control siRNA and Nedd4-2 siRNA were purchased from Santa Cruz Biotechnology. The hERG channel point mutation Y1078A and C-terminal truncation mutation Δ 1073 with disrupted Nedd4-2 binding sites were generated using PfuUltra Hotstart PCR Master Mix (Agilent Technologies, Santa Clara, CA). The mutations were confirmed by DNA sequencing (Eurofins MWG Operon, Huntsville, AL). The stable cell lines expressing mutant hERG channels were created in HEK 293 cells. Cells stably expressing wild type (WT) and mutant hERG channels were maintained in normal culture medium which contained minimum essential medium (MEM, Life Technology) supplemented with 1% non-essential amino acids and sodium pyruvate, 10% fetal bovine serum (FBS) and 0.4 mg/ml G418 (Invitrogen). For transfections, 2 μ g or 8 μ g of plasmids of interest were transfected into stable hERG-expressing HEK (hERG-HEK) cells grown in a 35-mm or a 100-mm dish, respectively, using Lipofectamine 2000 (Invitrogen). For electrophysiological analysis, a green fluorescent protein (GFP) plasmid (pIRES2-EGFP; Clontech) was co-transfected with the plasmid of interest at a ratio of 1:3 to identify the transfected cells. After transfection, cells were

cultured in normal medium for 24 h prior to experiments. To study the effect of PKA and PKC activations, hERG-HEK cells were incubated at 37 °C to the confluence of 60-80%. Cells were treated with or without the PKA activators (50 µM CPT-cAMP, 50 µM forskolin/FSK, 200 µM 3-isobutyl-1-methylxanthine/IBMX) or inhibitor (100 µM H89) and PKC activator (10 nM phorbol 12-myristate 13-acetate/PMA) or inhibitor (10 µM chelerythrine chloride/CE)

2. Neonatal rat cardiomyocyte isolation

Experimental protocols used for animal studies were approved by the Queen's University Animal Care Committee. Single ventricular myocytes were isolated from 1- to 2-day-old Sprague-Dawley rats of either sex by enzymatic dissociation as described previously (Guo *et al.*, 2007). Cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium (Invitrogen, Burlington, OH) supplemented with 10% FBS. Cells were cultured on coverslips and electrophysiological recordings were performed 24 h after transfections.

3. Co-immunoprecipitation (co-IP)

For each co-IP sample, 0.5 mg whole-cell proteins in 0.5 ml lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate/SDS, and 2mM EDTA in 1×PBS, pH 7.4) were incubated with the appropriate primary antibodies at 4 °C overnight. The protein complexes were mixed with protein A/G plus agarose beads (Santa Cruz Biotechnology) at 4 °C for 4 h and then precipitated by centrifugation at 10,000 × g for 1 min. The beads were washed 3 times with ice-cold lysis buffer followed by resuspension in 2×Laemmli sample buffer (for a total volume of 9.5 ml: 3.55 ml deionized water, 1.25 ml 0.5 M Tris-HCl at pH 6.8, 2.5 ml glycerol, 2.0 ml 10% w/v SDS, and 0.2 ml 0.5% w/v bromophenol blue) with 5% β-mercaptoethanol. The samples were boiled for 5 min and centrifuged at 10,000 × g for 10 min.

The supernatant was collected for Western blot analysis to detect proteins associated with the pull-down protein.

4. Cell-surface protein biotinylation and extraction

Twenty-four hours after transfection with pcDNA3, Ndfip1, or Ndfip2, the surface proteins of WT or Y1078A mutant hERG-HEK cells were extracted using the Pierce Cell Surface Protein Isolation Kit (Thermo Scientific). In brief, surface proteins from cells grown to 90-95% confluence were labelled with Sulfo-NHS-SS-Biotin at 4 °C for 30 min. Quenching solution was added to stop the labelling reaction. Cells with biotin-labelled surface proteins were collected and lysed. Biotin-labelled surface proteins were isolated using NeutrAvidin agarose columns and eluted with SDS-PAGE sample buffer (for total volume of 10 ml: 62.5 mM Tris-HCl at pH 6.8, 1 ml 10% SDS, and 1 ml 99% glycerol) containing 50 mM dithiothreitol (DTT), which releases the agarose-bound membrane proteins. Isolated cell-surface proteins were then analyzed by Western blot.

5. Proteinase K (PK) – cycloheximide (CHX) experiment

Twenty-four hours after transfection with pcDNA3, Ndfip1, or Ndfip2, intact WT or Y1078A mutant hERG-HEK cells were treated with proteinase K (PK, 200 µg/ml) at 37 °C for 20 min in cell culture dishes. Proteinase K was dissolved in a solution containing 10 mM HEPES, 150 mM NaCl, and 2 mM CaCl₂ (pH 7.4). The reaction was terminated by adding ice-cold phosphate buffered saline (PBS; every liter of PBS contains: 8.0 g NaCl, 0.2 g KCl, 0.24 g KH₂PO₄, and 1.44 g Na₂HPO₄ with pH adjusted to 7.4) containing 6 mM PMSF and 25 mM EDTA. Treated cells were washed and cultured in normal medium containing 10 µg/ml cycloheximide (CHX). Cells were then collected at various time points (0, 3, 6, 12, and 24 h), and whole-cell lysates were extracted for Western blot analysis.

6. Western blot analysis

Whole-cell (15 μ g) or surface proteins (200 μ g) in total volumes of 50 μ l from hERG-HEK cells after transfection with pcDNA3, human Nedd4-2, Ndfip1 or Ndfip2 plasmid, scrambled (control) siRNA, or Nedd4-2 siRNA, or after PKA and PKC activations or inhibitions were extracted and separated using 8% or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. Proteins in gels were transferred to polyvinylidene difluoride (PVDF) membranes and blocked for 1 h with 5% non-fat milk in 1 \times Tris-buffered saline (TBS, 0.1 M Tris chloride and 1.5 M NaCl in double distilled water) supplemented with 0.1% TWEEN 20. Membranes were incubated with appropriate primary antibodies and the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies each for 1 h. An enhanced chemiluminescent (ECL) kit (GE Healthcare) was used to visualize the blots on Fuji X-ray films. The expression level of actin (whole-cell) or Na⁺/K⁺ ATPase (surface protein) was used as a loading control. The band intensities were analyzed by normalizing each band to its corresponding actin or Na⁺/K⁺ ATPase and then to that of control cells. Band intensities are expressed as relative values to their corresponding controls.

7. Immunofluorescence microscopy

hERG-HEK cells were transfected with Myc-tagged human Ndfip1 or Ndfip2 plasmid. Twenty-four hours after transfection or incubation, the cells were fixed with freshly prepared ice-cold PBS containing 4% paraformaldehyde for 15 min. The fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min and blocked with 5% bovine serum albumin (BSA) in PBS for 1 h. For detecting cellular localization of Ndfip1 and Ndfip2, a rabbit anti-Myc primary antibody and Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody were used to label transfected Ndfip1 or Ndfip2 protein. The Golgi apparatus or MVBs were labelled with mouse

anti-58K Golgi protein (58K-9) or mouse anti-CHMP3 (charged multivesicular body protein 3) primary antibody, and the Alexa Fluor 594-conjugated donkey anti-mouse secondary antibody. For detecting the effects of Ndfip1 and Ndfip2 on hERG and Nedd4-2 protein expression, the goat anti-hERG (C-20) primary antibody and Alexa Fluor 488-conjugated donkey anti-goat secondary antibody were used to visualize hERG proteins; the mouse anti-Myc primary antibody and Alexa Fluor 594-conjugated donkey anti-mouse secondary antibody were used to label transfected Ndfip1 or Ndfip2 protein; the rabbit anti-Nedd4L primary antibody and the Alexa Fluor 633-conjugated goat anti-rabbit secondary antibody were used to stain Nedd4-2. Cells were incubated with primary and secondary antibodies each for 1 h. ProLong Gold Antifade reagent (Life Technologies) was used as the mounting solution. Images were acquired using a Leica TCS SP2 Multi Photon confocal microscope (Leica, Germany). The high quality fluorescence objective lenses used was HCX PL APO DIC 63X/1.20 W. Corr/0.17 CS. The three filter tubes used are I3 (450 nm – 490 nm excitation, with dichroic mirror at 510 nm), N2.1 (515 nm – 560 nm excitation, with dichroic mirror at 580 nm), and A (LP emission at 590 nm). The green, red, and blue fluorescent signals were generated using solid state lasers for 488 nm, 594 nm, and 633 nm excitations, respectively.

8. Patch-clamp recordings

The whole-cell patch-clamp method was used. Pipette resistance was between 1.5 M Ω and 4.5 M Ω . Series resistance was between 3 M Ω and 10 M Ω . hERG-HEK cells were held at –80 mV. hERG current (I_{hERG}) was elicited by depolarizing steps to voltages between –70 mV and 70 mV in 10-mV increments. The depolarizing steps were followed by a repolarizing step to –50 mV to induce tail currents. The tail current amplitude following the 50 mV depolarization step was used for analysis of I_{hERG} . I_{hERG} was sampled at 1 kHz and filtered at 5 kHz. The bath

solution contained 135 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES (pH 7.4 with NaOH). The pipette solution contained 135 mM KCl, 5 mM EGTA, 1 mM MgCl₂, and 10 mM HEPES (pH 7.2 with KOH). The cultured neonatal rat ventricular myocytes were held at -80 mV, and the Cs⁺-mediated I_{Kr} was evoked by depolarizing pulses to voltages between -70 and 70 mV in 10-mV increments. Tail current upon repolarization to the holding potential of -80 mV after the depolarizing pulse to 50 mV was used for analyzing current amplitudes. I_{Kr} was sampled at 20 kHz and filtered at 5 kHz. The bath solution contained 135 mM CsCl, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES, and 10 μM nifedipine (pH 7.4 with CsOH). The pipette solution contained 135 mM CsCl, 5 mM MgATP, 10 mM EGTA, and 10 mM HEPES (pH 7.2 with CsOH). In both hERG-HEK cells and neonatal rat ventricular myocytes, the tail currents were normalized to the corresponding cell-capacitance (10 – 15 pF) and were expressed as current densities (pA/pF). All patch-clamp experiments were performed at room temperature (22±1 °C).

9. Reagents and antibodies

MEM, FBS, non-essential amino acids, sodium pyruvate, Alexa Fluor 633-conjugated goat anti-rabbit, Alexa Fluor 594-conjugated donkey anti-mouse, anti-rabbit, Alexa Fluor 488-conjugated goat anti-rabbit and donkey anti-goat secondary antibodies were purchased from Invitrogen. PKA (CPT-cAMP, FSK, IBMX and H89) and PKC (PMA and CE) activators and inhibitors, proteinase K (PK), cycloheximide (CHX), Rabbit anti-Kv11.1 (hERG), mouse anti-HA, anti-actin, and anti-CHMP3 primary antibodies, G418, electrolytes, EDTA, EGTA, HEPES, glucose, PMSF and BSA were purchased from Sigma-Aldrich. Mouse anti-Ndfip1, goat anti-Ndfip2 and anti-hERG (C-20), rabbit anti-Myc primary antibodies, as well as goat anti-mouse, mouse anti-goat, goat anti-rabbit IgG-HRP secondary antibodies, and protein A/G beads were

purchased from Santa Cruz Biotechnology. Rabbit anti-Nedd4-2 antibody was purchased from Cell Signaling Technology. Mouse anti-58K Golgi protein (58K-9) antibody was purchased from Abcam Biotechnology.

10. Statistical analysis

All results are presented as the mean \pm the standard error of the mean (S.E.M). One-way analysis of variance, Newman-Keuls post hoc test, and an unpaired student's t-test were used to determine the statistical significance between the control and test groups. A *P* value of 0.05 or less was considered statistically significant.

Chapter 3: Results

1. Regulation of Nedd4-2-mediated hERG channel degradation by Ndfip1 and Ndfip2

1.1 Nedd4-2 regulates hERG channel function and mature hERG protein expression

We have previously demonstrated that Nedd4-2 targets the PY motif in the C-terminus of hERG channels to mediate hERG degradation, and the overexpression of Nedd4-2 decreases the 155-kDa hERG protein and hERG current (Guo *et al.*, 2012). To investigate the role of endogenous Nedd4-2 on the homeostasis of hERG channel expression and function, hERG-HEK cells were transfected with control (Ctrl) or Nedd4-2 siRNA to knock down Nedd4-2. Compared to the hERG-HEK cells transfected with Ctrl siRNA (n=12), those transfected with Nedd4-2 siRNA (n=11) displayed an increased I_{hERG} (Figure 4A, $P<0.05$) and 155-kDa hERG protein expression (Figure 4B, $P<0.01$, n=4). In contrast, transfection of Nedd4-2 siRNA to HEK cells stably expressing the Nedd4-2-interaction-deficient mutant hERG channel Y1078A did not affect hERG current (Figure 4C) or protein expression (Figure 4D). Thus, Nedd4-2 regulates hERG channel function via modulating mature protein expression.

1.2 Ndfip1 and Ndfip2 interact with Nedd4-2 as well as hERG protein

Nedd4-2 targets the PY motif (PPAY) in the intracellular C-terminus of hERG channels to mediate protein degradation (Guo *et al.*, 2012). Although both the 135-kDa and 155-kDa forms of hERG protein contain PY motif, only the 155-kDa form is targeted by Nedd4-2. This observation suggests additional molecules being involved in the hERG-Nedd4-2 interaction. Like other Nedd4 family proteins, Nedd4-2 is under constant regulation by various molecules (Cui & Zhang, 2013; Lamothe & Zhang, 2013; Wang *et al.*, 2014). In particular, Nedd4-2 also possesses a PY (LPXY) motif in its catalytic HECT domain (Bruce *et al.*, 2008). In the absence

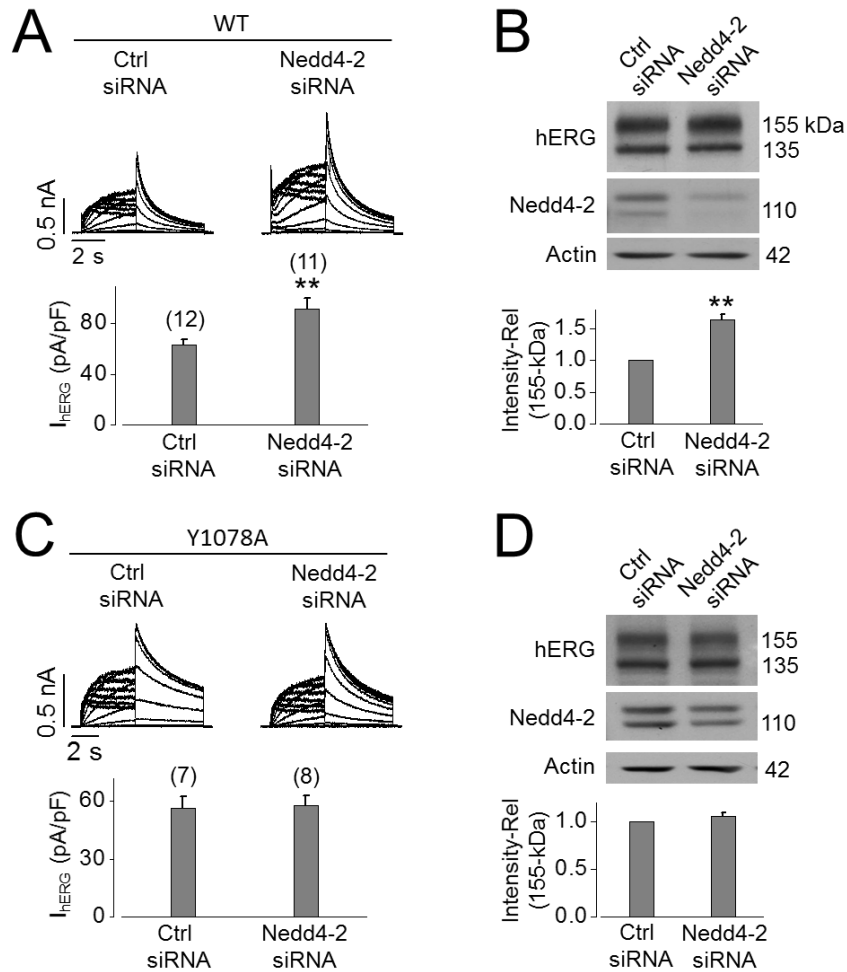


Figure 4: Nedd4-2 regulates hERG channel function and expression.

(A and C) Knockdown of Nedd4-2 enhances hERG current in wild type (WT) (A) but not Y1078A mutant (C). Families of hERG currents (upper panel) recorded from cells transfected with scrambled control (Ctrl) siRNA or Nedd4-2 siRNA. I_{hERG} at 50 mV depolarization voltage was used for quantification and are summarized in the bar graph (lower panel). The numbers above bars indicate the numbers of cells tested in three independent experiments. (B and D) Knockdown of Nedd4-2 increases the protein expression of mature WT (B) but not Y1078A mutant (D) hERG channels. The intensity of the 155-kDa hERG band from cells transfected with Nedd4-2 siRNA was normalized to the value from those transfected with Ctrl siRNA and summarized in the bar graph below the Western blot images (n=3 or 4). Actin expression was used as a loading control. Error bar, S.E. * $P < 0.05$, ** $P < 0.01$.

of other substrates, the PY motif of Nedd4-2 weakly binds to its own WW domains, which serves to stabilize Nedd4-2. Ndfip1 and Ndfip2 are among those interacting with the WW domains of Nedd4 family proteins, both of which are endogenously expressed in HEK293 cells and the human heart (Harvey *et al.*, 2002; Konstas *et al.*, 2002). By binding to the WW domains of Nedd4-2, Ndfip1 and Ndfip2 release the catalytic HECT domain, thereby regulating Nedd4-2 activity. My co-IP analyses show that both Ndfip1 and Ndfip2 interact with Nedd4-2. As shown in the upper panels of Figure 5A and B, Nedd4-2 expression was detected in proteins precipitated with anti-Ndfip1 or anti-Ndfip2 antibody from the whole-cell lysate of Ndfip1- or Ndfip2-transfected hERG-HEK cells, respectively. Conversely, Ndfip1 or Ndfip2 expression was detected in proteins precipitated with the Nedd4-2 antibody (Figure 5A and B, lower panels).

We have shown that Nedd4-2 interacts with hERG channels (Guo *et al.*, 2012). Since we demonstrated that Nedd4-2 interacts with Ndfip1 and Ndfip2, I investigated whether Ndfip1 or Ndfip2 forms a 3-component complex with Nedd4-2 and hERG channels. My co-IP analysis shows that when Ndfip1 or Ndfip2 was immunoprecipitated in WT hERG-HEK cells, the 155-kDa hERG protein band was detected (Figure 6A). However, neither Ndfip1 nor Ndfip2 immunoprecipitated with the $\Delta 1073$ mutant hERG channels in which the Nedd4-2 recognition site is removed (Figure 6B and C).

1.3 Overexpression of Ndfip1 or Ndfip2 decreases hERG channel current via Nedd4-2

Ndfip1 and Ndfip2 both interact with Nedd4-2 which mediates hERG channel degradation. To study the effect of Ndfip1 or Ndfip2 on hERG channel function, we overexpressed Ndfip1 or Ndfip2 in hERG-HEK cells. Twenty-four hours after transfection, I_{hERG} was recorded using the whole-cell patch-clamp method. As shown in Figure 7A, Ndfip1 or Ndfip2 overexpression significantly decreased I_{hERG} ($P < 0.01$, $n = 13-20$ cells). However, the half activation voltage and

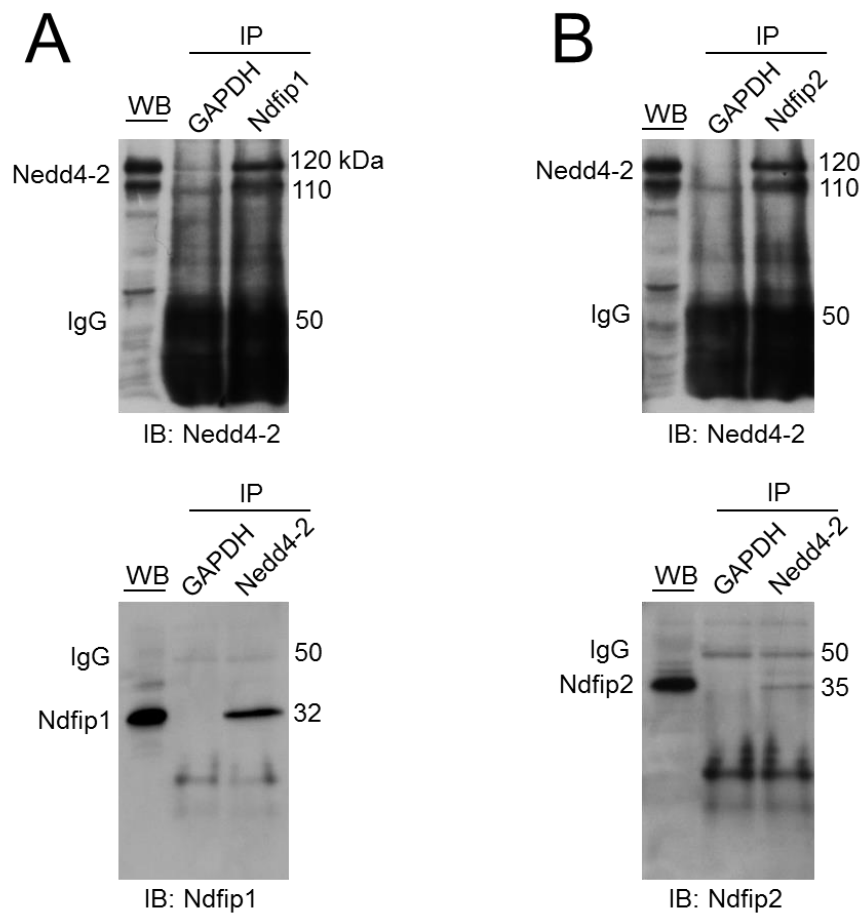


Figure 5: Ndfip1 and Ndfip2 both interact with Nedd4-2.

hERG-HEK cells were transfected with Ndfip1 (A) or Ndfip2 (B) plasmid (n=3). Whole-cell lysates were precipitated with goat anti-GAPDH or anti-Ndfip1 (A, upper panel), mouse anti-GAPDH or anti-Ndfip2 (B, upper panels), rabbit anti-GAPDH or anti-Nedd4-2 (lower panels) antibody. The precipitates were immunoblotted with rabbit anti-Nedd4-2 primary and goat anti-rabbit IgG HRP secondary antibodies (upper panels), goat anti-Ndfip1 primary and mouse anti-goat IgG HRP secondary antibodies (A, lower panel) or mouse anti-Ndfip2 primary and goat anti-mouse secondary antibodies (B, lower panel), respectively.

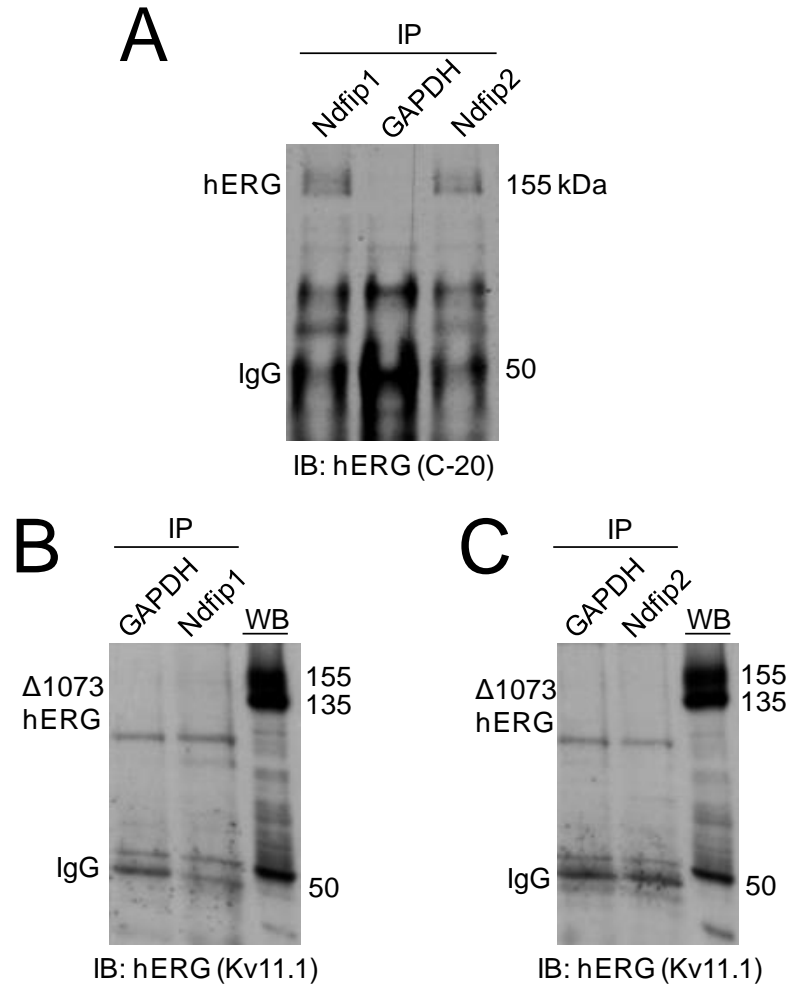


Figure 6: Ndfip1 and Ndfip2 interact with the WT 155-kDa hERG but not the Δ1073 mutant.

(A) Whole-cell lysates of Ndfip1 or Ndfip2-transfected WT hERG-HEK cells were precipitated with anti-GAPDH, anti-Ndfip1 or anti-Ndfip2 antibody, and immunoblotted with anti-hERG (C-20) hERG antibody (n=3). (B and C) Whole-cell lysates of Ndfip1- or Ndfip2-transfected Δ1073 hERG-HEK cells were precipitated with anti-GAPDH, anti-Ndfip1 or anti-Ndfip2 antibody, and were immunoblotted with anti-Kv11.1 (hERG) antibody (n=3). WT hERG-HEK cells were used as the positive control on the right.

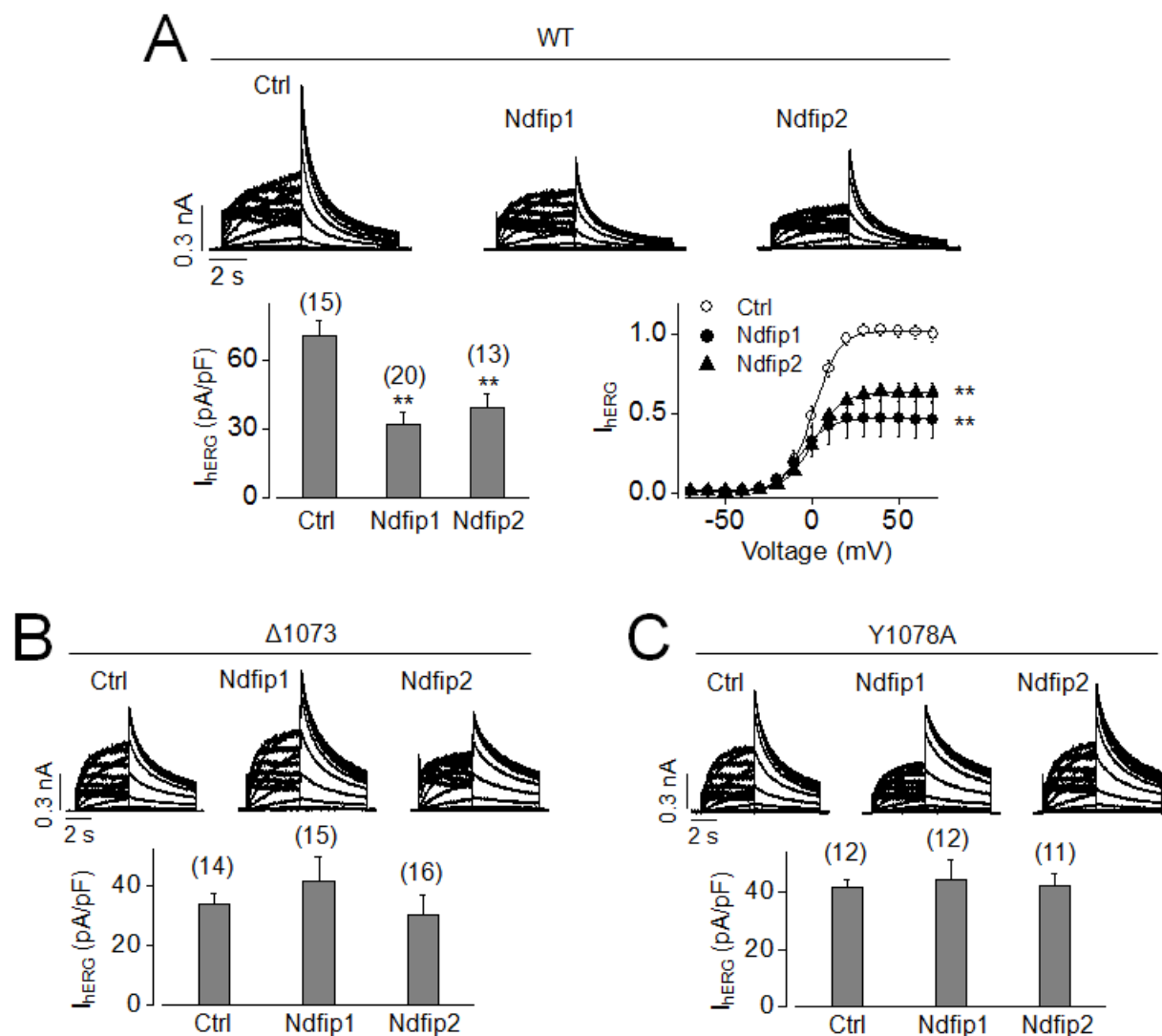


Figure 7: Overexpression of Ndfip1 or Ndfip2 decreases WT hERG current, but not the Nedd4-2-interaction deficient mutant hERG currents.

Families of WT (A), $\Delta 1073$ (B) or Y1078A (C) hERG tail currents recorded from hERG-HEK cells 24 h after transfection with pcDNA3 (Ctrl), Ndfip1, or Ndfip2. I_{hERG} at 50-mV depolarization voltage was normalized to the corresponding cell capacitance and summarized in bar graphs. Activation curves for each group are shown for WT hERG-HEK cells in (A). (** $P < 0.01$ from 10 mV to 70 mV, $n = 5-7$ cells for each group). The numbers above bars indicate the numbers of cells tested from three independent experiments. Error bar, S.E.

the slope factor were not affected by either Ndfip1 or Ndfip2 overexpression. Specifically, the half activation voltages and slope factors were 1.1 ± 0.4 mV and 6.8 ± 0.4 mV in control cells, -2.4 ± 0.9 mV and 7.0 ± 0.6 mV in Ndfip1-transfected cells ($P > 0.05$, $n = 4-6$ cells), and 0.4 ± 0.7 mV and 7.1 ± 0.5 mV in Ndfip2-transfected cells ($P > 0.05$, $n = 5-7$ cells).

To confirm that Ndfip1- and Ndfip2 reduce I_{hERG} through Nedd4-2, the effect of Ndfip1 or Ndfip2 on the $\Delta 1073$ or Y1078A mutant hERG channels was examined. The C-terminal truncation mutant $\Delta 1073$ removes, whereas the point mutation Y1078A, disrupts the PY motif in the hERG channel where Nedd4-2 binds. We have previously shown that both of these mutations eliminate Nedd4-2-mediated hERG channel reduction (Guo *et al.*, 2012). My data now show that these two mutations also eliminated the Ndfip1- and Ndfip2-mediated I_{hERG} reduction (Figure 7B and C), indicating that both Ndfip1 and Ndfip2 decrease I_{hERG} via Nedd4-2.

1.4 Ndfip1 and Ndfip2 decrease hERG protein on the plasma membrane

Overexpression of either Ndfip1 or Ndfip2 decreases I_{hERG} (Figure 7A). To examine whether a reduction in the 155-kDa hERG protein is responsible for the Ndfip1- or Ndfip2-mediated I_{hERG} decrease, I transfected Ndfip1 or Ndfip2 plasmid into HEK cell lines stably expressing WT or Y1078A mutant hERG channels, and performed Western blot analysis using whole-cell lysates. Ndfip1 overexpression decreased the 155-kDa WT hERG protein by 29.9% ($P < 0.01$, $n = 4$), but did not affect the Y1078A hERG channel expression (Figure 8A). These data indicate that Ndfip1 reduces the 155-kDa hERG protein expression through Nedd4-2. Surprisingly, although Ndfip2 overexpression significantly decreased I_{hERG} , it did not affect the 155-kDa hERG protein expression level. As expected, Ndfip2 also did not affect the protein expression of the Nedd4-2 interaction-deficient mutant hERG channel Y1078A (Figure 8B, $P > 0.05$, $n = 4$).

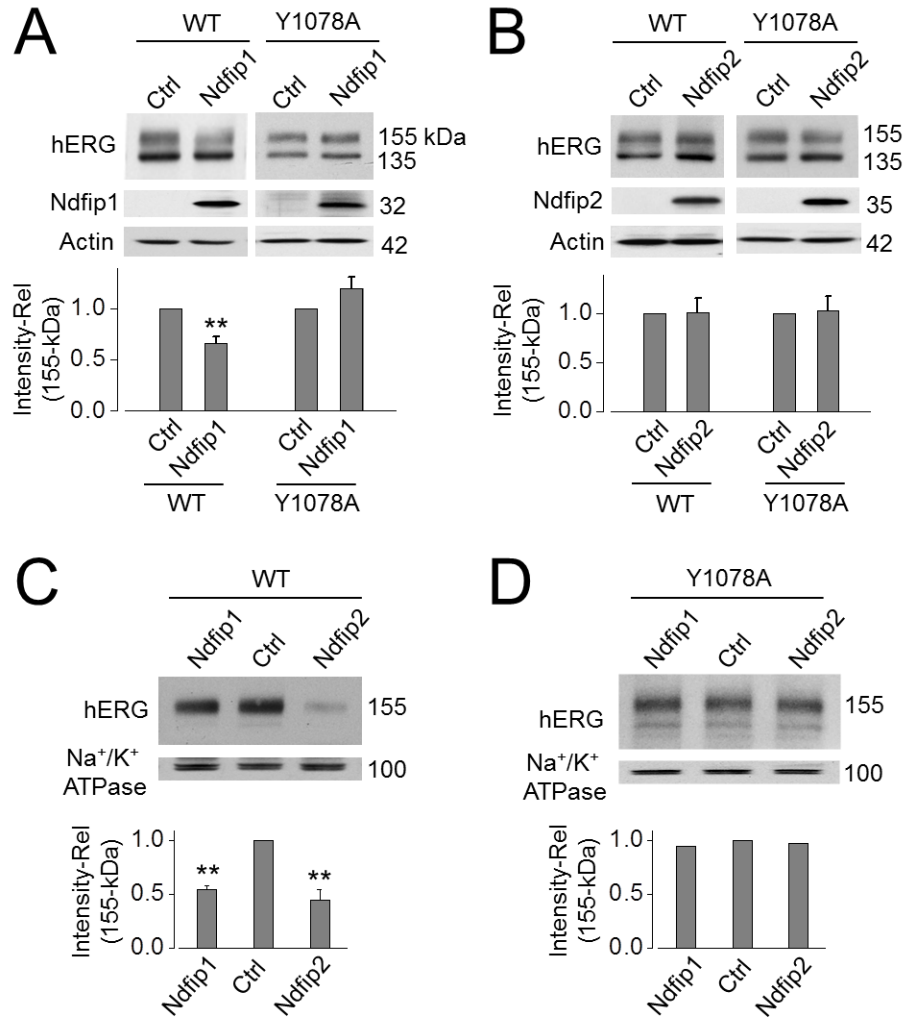


Figure 8: *Ndfip1* and *Ndfip2* decrease only WT hERG surface expression

(A and B) The effects of *Ndfip1* and *Ndfip2* on the expression of WT or Y1078A hERG whole-cell protein. WT or Y1078A hERG-HEK cells were transfected with pcDNA3 (Ctrl), *Ndfip1*, or *Ndfip2* plasmid. Whole-cell lysates were collected 24 h after transfection. Actin expression was used as a loading control. (C and D) The effects of *Ndfip1* and *Ndfip2* on the expression of cell-surface WT or Y1078A hERG proteins. HEK cells stably expressing WT or Y1078A hERG channels were transfected with pcDNA3 (Ctrl), *Ndfip1* or *Ndfip2* plasmid. Twenty-four hours after transfection, cell-surface proteins isolated using biotinylation were analyzed using Western blot. Protein expression of Na⁺/K⁺ ATPase was used as a loading control. Error bar, S.E. ** $P < 0.01$, n=3-5.

To investigate the discrepancy between Ndfip2-mediated I_{hERG} reduction and the unaltered 155-kDa hERG expression in whole-cell lysate, I isolated cell-surface proteins using biotinylation and examined the effects of Ndfip1 and Ndfip2 on surface-expressed hERG channels using Western blot analysis. Ndfip1 and Ndfip2 significantly reduced the cell-surface hERG protein (Figure 8C, $P < 0.01$, $n = 5$). Furthermore, neither Ndfip1 nor Ndfip2 overexpression affected the cell-surface expression of Y1078A mutant hERG protein (Figure 8D). Therefore, the decreased I_{hERG} induced by Ndfip1 or Ndfip2 overexpression is a result of reduced 155-kDa hERG protein expression on the plasma membrane.

1.5 Ndfip1 is localized in the Golgi apparatus while Ndfip2 is mainly present in the MVBs

The decreased cell-surface hERG channels with an unchanged total 155-kDa hERG protein implies an increased intracellular 155-kDa hERG protein in Ndfip2-transfected cells. The different effects of Ndfip1 and Ndfip2 on whole-cell 155-kDa hERG protein expression led me to hypothesize that they act through distinct pathways. To test this hypothesis, I investigated the cellular localization of Ndfip1 and Ndfip2. Consistent with the literature, Figure 9 shows that Ndfip1 is located in the Golgi apparatus. On the other hand, while some Ndfip2 is also present in the Golgi apparatus, it is primarily located in the MVBs.

I further examined hERG expression and localization in hERG-HEK cells transfected with or without Ndfip1 or Ndfip2. Compared with untransfected cells, those transfected with Ndfip1 showed reduced hERG expression (Figure 10A) while such an effect was not present in the Y1078A mutant cell line (Figure 10C). In contrast to hERG-HEK cells transfected with Ndfip1, those with Ndfip2 primarily displayed an intracellular accumulation of hERG which colocalized with Ndfip2 and Nedd4-2 (Figure 10B), and this effect was absent in cells expressing Y1078A mutant hERG channels (Figure 10D).

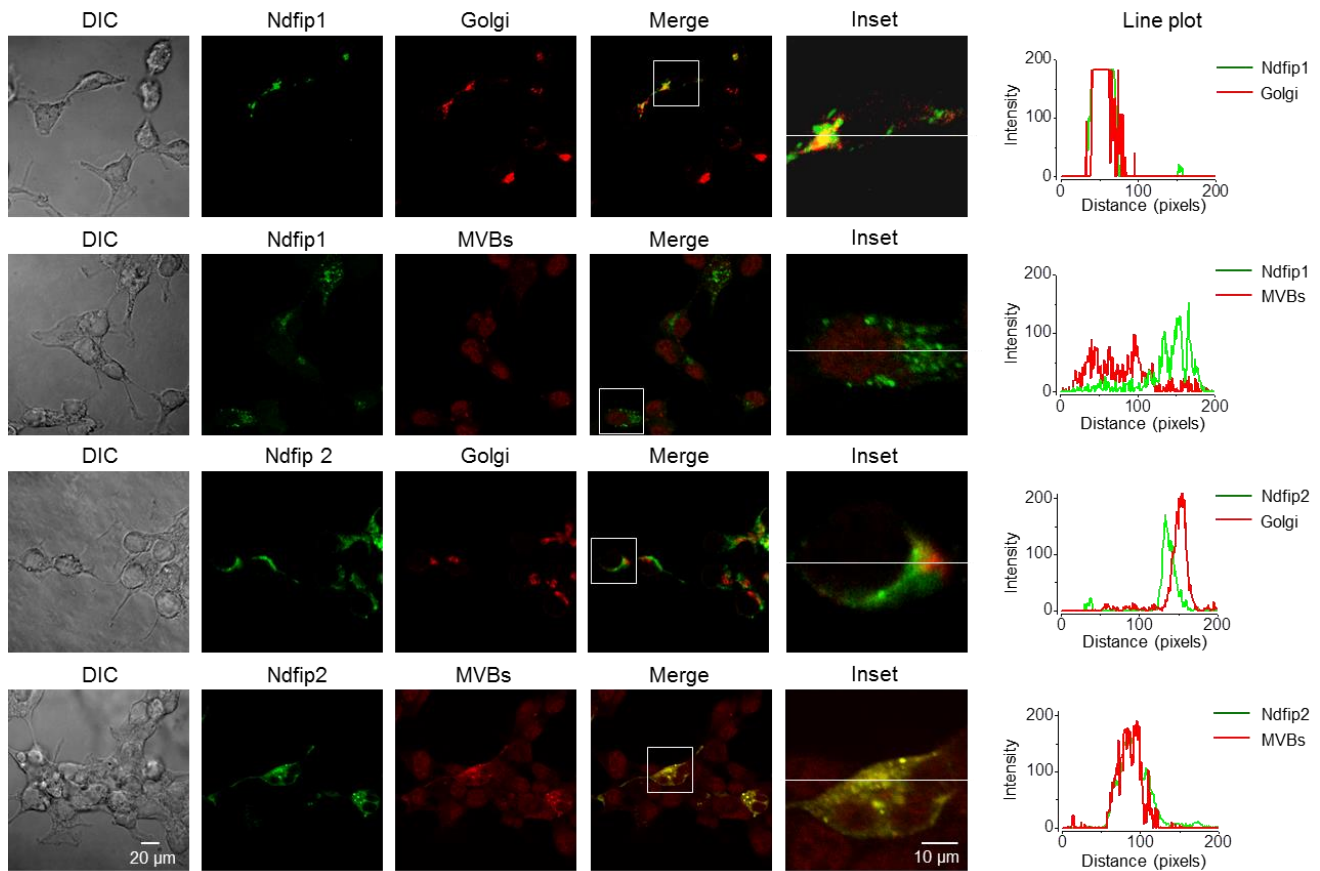


Figure 9: *Ndfip1* is localized in the Golgi apparatus while *Ndfip2* is localized primarily in the multivesicular bodies (MVBs).

Confocal images illustrating that the transfected *Ndfip1* colocalizes with the Golgi apparatus while transfected *Ndfip2* colocalizes with the CHMP3-marked MVBs, and to a lesser extent with Golgi apparatus. hERG-HEK cells were transfected with Myc-tagged *Ndfip1* or *Ndfip2*. Cells were fixed and permeabilized 24 h after transfection. *Ndfip1* or *Ndfip2* was stained with rabbit anti-Myc primary antibody and Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (green). The Golgi apparatus or MVBs were labeled with mouse anti-58K Golgi protein or mouse anti-CHMP3 primary antibody and Alexa Fluor 594-conjugated donkey anti-mouse secondary antibody (red). Cells are indicated in DIC images on the left, and those selected for quantification are indicated by white squares. Intensities of the fluorescence signals along lines across the images were quantified by ImageJ and shown in the right panels.

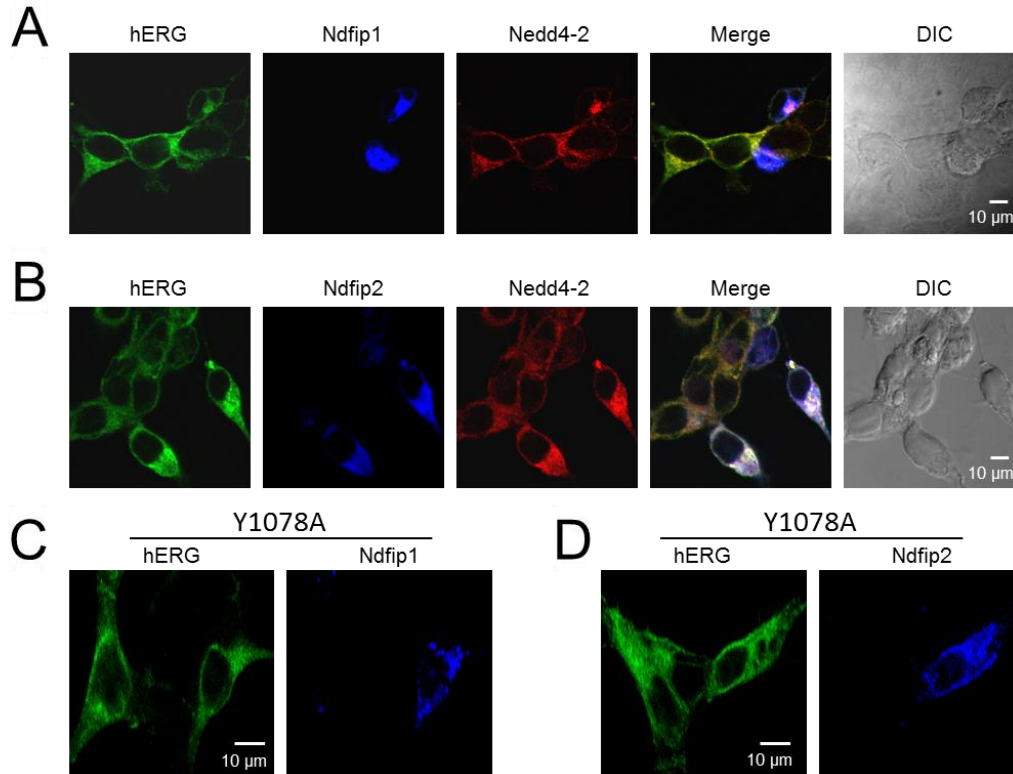


Figure 10: Effects of Ndfip1 and Ndfip2 on the expression pattern of WT and Nedd4-2-interaction-deficient Y1078A mutant hERG channels.

(A) Ndfip1 overexpression reduced the protein level of WT hERG channels. (B) Ndfip2 caused intracellular accumulation of hERG channels. (C and D) The overexpression of either Ndfip1 (C) or Ndfip2 (D) did not affect the expression of the Y1078A mutant hERG channels. In (A) to (D), cells were transfected with Myc-tagged Ndfip1 or Ndfip2, and stained with mouse anti-Myc primary and Alexa Fluor 594-conjugated donkey anti-mouse secondary antibodies (blue). hERG was labeled with goat anti-hERG (C-20) primary and Alexa Fluor 488-conjugated donkey anti-goat secondary antibodies (green). In both (A) and (B), Nedd4-2 was stained using rabbit anti-Nedd4-2 primary and Alexa Fluor 633-conjugated goat anti-rabbit secondary antibodies (red).

1.6 Both *Ndfip1* and *Ndfip2* interfere with hERG channel maturation

To study the effect of *Ndfip1* and *Ndfip2* on hERG channel forward trafficking and degradation more directly, I performed a modified cycloheximide chase experiment. WT or Y1078A mutant hERG-HEK cells were transfected with pcDNA3, *Ndfip1*, or *Ndfip2* plasmid. Twenty-four hours after transfection, cells were treated with proteinase K (PK, 200 µg/ml) for 20 min to clear the cell-surface expressed 155-kDa hERG channels (Zhou *et al.*, 1998; Rajamani *et al.*, 2006). Cells with 155-kDa hERG protein removed by PK-treatment were then cultured in the normal medium with cycloheximide (CHX, 10 µg/ml) to inhibit protein synthesis. Cells were collected at different time points after culture (0, 3, 6, 12, and 24 h), and whole-cell proteins were analyzed by Western blot to examine the reappearance of the upper 155-kDa hERG protein band as a result of maturation of the existing 135-kDa hERG protein band prior to cycloheximide application. As shown in Figure 11A, in the control (pcDNA3) group, the 155-kDa band was completely removed by PK digestion (Figure 11A, 0 h). The 155-kDa band gradually reappeared with time, reflecting the maturation of the 135-kDa hERG channels. It reached maximum expression at 12 h after PK digestion and then reduced with time, reflecting the degradation of hERG channel proteins. In the *Ndfip1*-transfected group, the 155-kDa band was also completely removed by PK digestion (Figure 11A, 0 h). The maturation of the 135-kDa to the 155-kDa hERG channel was slightly slower than that of control, and the 155-kDa band is present at lower levels at each time point of the chase in both *Ndfip1*- and *Ndfip2*-transfected hERG-HEK cells. Unlike the pcDNA3- and *Ndfip1*-transfected cells, the PK treatment did not completely eliminate the 155-kDa band at the 0 h time point in *Ndfip2*-transfected cells. *Ndfip2* also enhanced hERG channel degradation during maturation, leading to smaller amounts of 155-kDa hERG expression

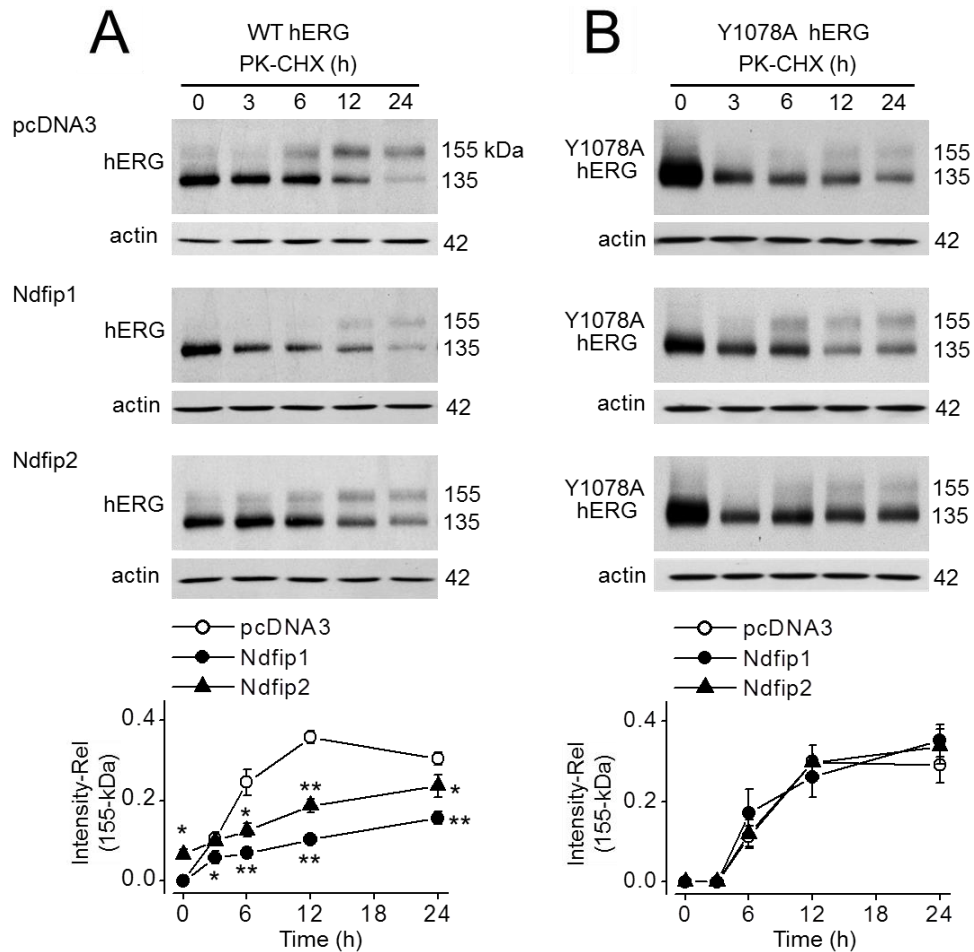


Figure 11: Effects of Ndfip1 and Ndfip2 on WT and Y1078A mutant hERG maturation.

Ndfip1 and Ndfip2 decrease the expression of the 155-kDa form of WT hERG channels (A) during maturation of the channel from the 135-kDa to the 155-kDa form, but did not affect that of Y1078A mutant hERG channel (B). WT or Y1078A hERG-HEK cells were transfected with pcDNA3, Ndfip1 or Ndfip2 plasmid. Twenty-four hours after transfection, the 155-kDa cell-surface hERG channels were removed by proteinase K treatment (PK, 200 μ g/ml, 20 min). Treated cells were then incubated in medium containing cycloheximide (CHX, 10 μ g/ml) to inhibit protein biosynthesis and were collected at various time points (0, 3, 6, 12, 24 h) for Western blot analysis. The 155-kDa band intensity at each time point was normalized to that of the 135-kDa band at time 0 h in the same gel, and summarized data were plotted against time points in the lower panels. Error bar, S.E. * $P < 0.05$, ** $P < 0.01$, $n = 3$ for each group.

than those in the control group. Furthermore, it appears that the overall degradation of hERG proteins was slowed by Ndfip2 transfection (Figure 11A). In contrast to WT hERG channels, maturation of the Nedd4-2-interaction-deficient Y1078A mutant hERG channels were not affected by either Ndfip1 or Ndfip2 overexpression (Figure 11B).

1.7 Ndfip1 and Ndfip2 decreases I_{Kr} in neonatal rat ventricular myocytes

Nedd4-2, Ndfip1 and Ndfip2 are widely expressed in mammalian cells (Harvey *et al.*, 2002; Konstas *et al.*, 2002). I performed patch-clamp experiments in neonatal rat ventricular myocytes to verify that the observations we made in cell lines also apply to cardiomyocytes. Overexpression of Nedd4-2 significantly reduced I_{Kr} , while knockdown of Nedd4-2 by siRNA transfection elevated I_{Kr} in cardiomyocytes (Figure 12A and B, $P < 0.01$, $n = 8-16$ cells). Moreover, overexpression of either Ndfip1 or Ndfip2 reduced I_{Kr} (Figure 12C, $P < 0.01$, $n = 15-19$ cells).

2. Regulation of Nedd4-2 mediated hERG channel degradation by PKA and PKC

2.1 PKA and PKC signalling alters hERG expression

To activate PKA, IBMX (200 μM) was used as phosphodiesterase inhibitor to reduce degradation of cAMP, causing increased cAMP levels, therefore presumably enhancing PKA activation. To inhibit the PKA function, cells were treated with H89 (100 μM), a competitive inhibitor of ATP, to block the conversion of ATP to cAMP by adenylyl cyclase, and to subsequently decrease PKA activity. Cells were incubated for 24 h with PKA activator or inhibitor. Figure 13A shows that the activation of PKA by IBMX significantly increased hERG channel expression in both the core- glycosylated 135-kDa and the fully glycosylated 155-kDa hERG bands ($P < 0.01$, $n = 3$). The inhibition of PKA by H89 reduced the fully glycosylated 155-kDa hERG channel expression significantly ($P < 0.05$, $n = 3$). Thus, PKA signalling may constitutively regulate hERG channel protein expression.

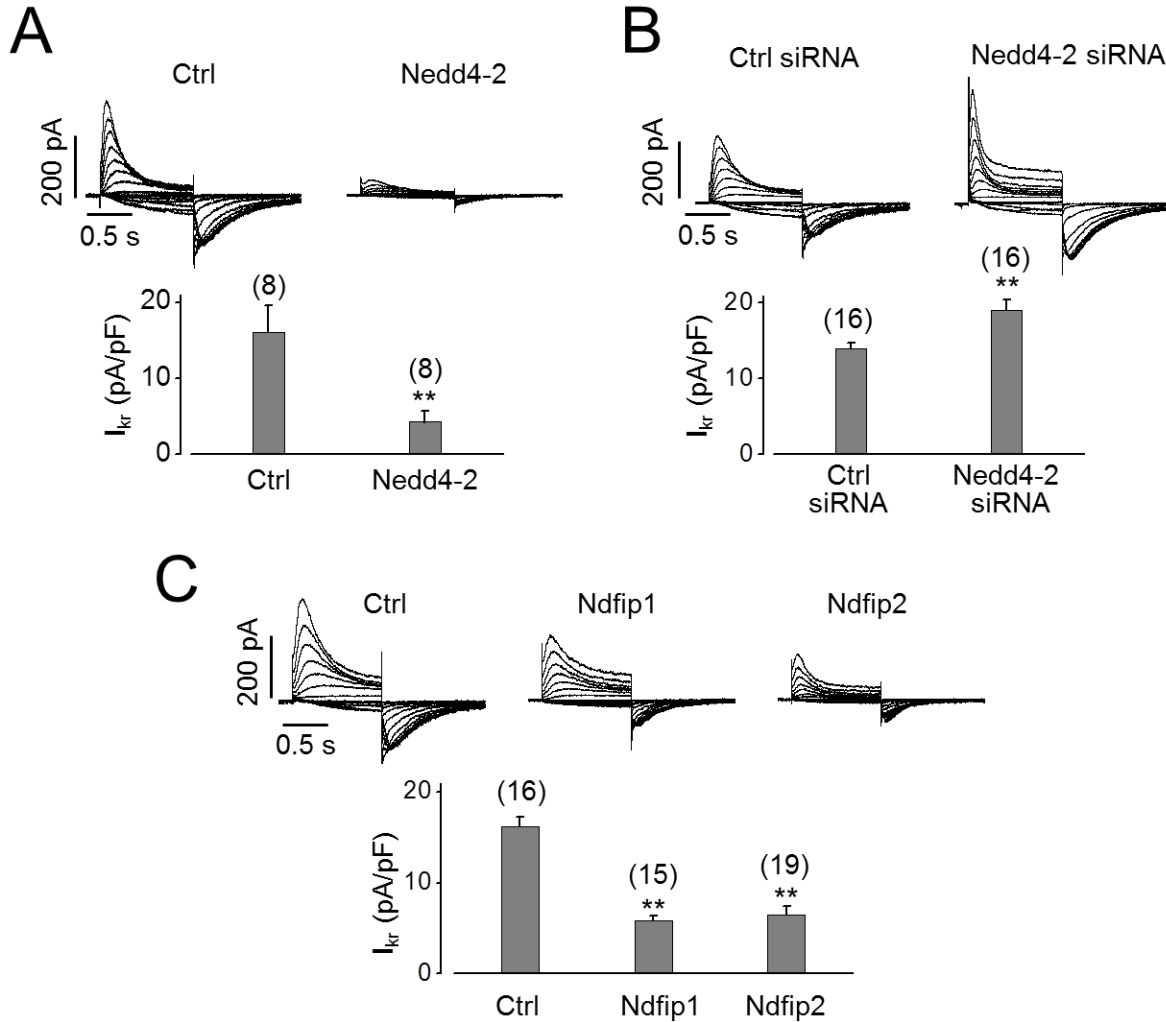


Figure 12: *Nedd4-2*, *Ndfip1* and *Ndfip2* regulate I_{Kr} in neonatal rat ventricular myocytes.

(A) Overexpression of *Nedd4-2* decreases I_{Kr} . (B) Knockdown of *Nedd4-2* elevates I_{Kr} . (C) Overexpression of *Ndfip1* or *Ndfip2* reduces I_{Kr} . In A-C, cultured neonatal rat ventricular myocytes were cotransfected with GFP plus pcDNA3 (Ctrl), or *Nedd4-2* plasmid; control (Ctrl) siRNA or *Nedd4-2* siRNA; pcDNA3 (Ctrl), *Ndfip1* or *Ndfip2* plasmid. Families of Cs^+ -mediated I_{Kr} in GFP-positive cells under each condition were recorded 24 h after transfection. The summarized I_{Kr} at depolarization voltage of 50 mV, which is normalized to cell capacitance, is shown below the current traces. The numbers above bars indicate the numbers of cells tested from at least three independent experiments. Error bar, S.E. * $P < 0.05$, ** $P < 0.01$.

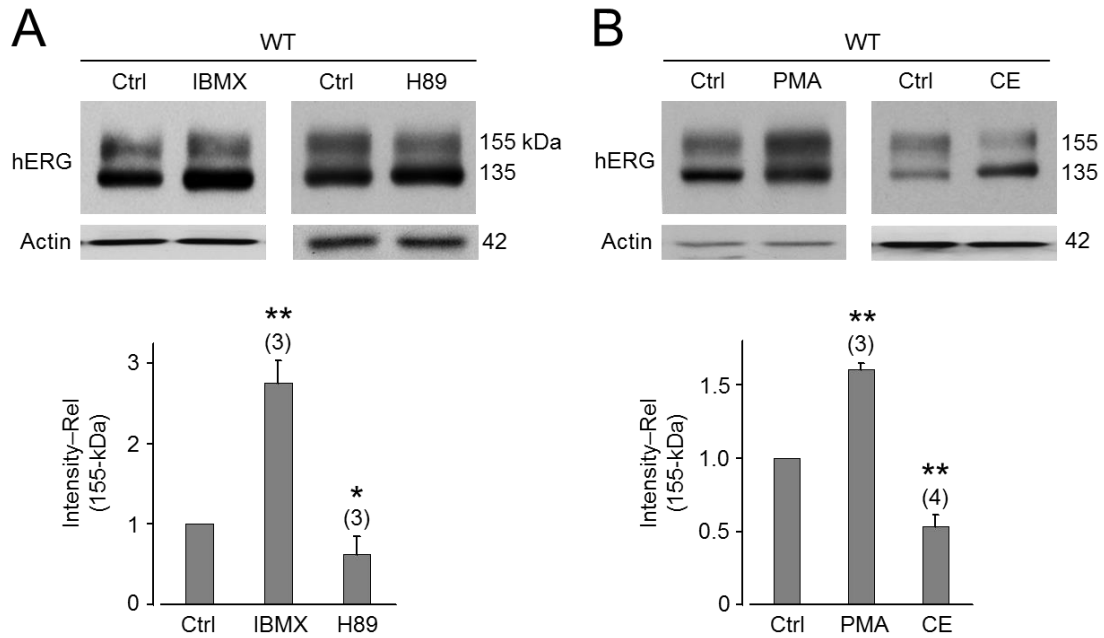


Figure 13: PKA and PKC activation independently elevate hERG protein expression.

(A) Upper panel: Western blot analysis showing hERG protein expression levels after treating hERG-HEK cells with PKA activator (200 μ M IBMX) or inhibitor (100 μ M H89) for 24 h. Lower panel: Relative intensity of 155-kDa band after treating cells with PKA activator (IBMX) or inhibitors (H89) compared to Ctrl (n =3). (B) Upper panel: Western blot analysis showing hERG protein expression levels after treating hERG-HEK cells with PKC activator (10 nM PMA) and inhibitor (10 μ M Chelerythrine, CE) for 24 h. Lower panel: Relative intensity of 155-kD band after treatments of hERG-HEK cells with PKC activator (PMA) and inhibitor (CE) comparing to Ctrl (n = 3 or 4). Actin expression was used as a loading control. Error bars, S.E. * $P < 0.05$, ** $P < 0.01$.

The DAG analog PMA (10 nM) was used to activate PKC, and chelerythrine chloride (CE, 10 μ M) was used to inhibit PKC. Cells were collected 24 h after incubating with PMA or CE. Figure 13B indicates that PMA-treated cells show significantly enhanced expression level of 155-kDa hERG protein ($P < 0.01$, $n = 3$). CE-treated cells show significantly reduced level of hERG expression ($P < 0.01$, $n = 4$). Therefore, PKC activation enhances while its inhibition reduces hERG expression.

2.2 The PKA- or PKC-mediated augmentation of hERG protein expression does not reveal in enhanced hERG current

To test the effect of PKA or PKC activation on hERG channel functionality, whole-cell patch-clamp analysis was used after hERG-HEK cells were treated with IBMX or PMA, respectively, for 24 h. Unexpectedly, the enhancement in hERG protein expression, especially the mature 155-kDa band, upon PKA or PKC activation was not accompanied by an increase in hERG current. The peak tail currents recorded from cells after PKA or PKC treatment did not show a significant increase comparing to control (Figure 14). Therefore, 24-h PKA or PKC activation only increases whole-cell hERG protein but not I_{hERG} .

2.3 The increased 155-kDa hERG by PKA and PKC activation are on the cell membrane

The discrepancy between an increased 155-kDa hERG band and unchanged current raised two possibilities. One is that the increased proteins are located inside the cell instead of being inserted in the plasma membrane, and the other is that the extra 155-kDa hERG channels on the cell membrane is inactivated by channel phosphorylation since multiple PKA and PKC phosphorylation sites exist in hERG proteins and channel phosphorylation have been shown to inhibit its function (Wei *et al.*, 2002; Cockerill *et al.*, 2007). To test whether the increased 155-

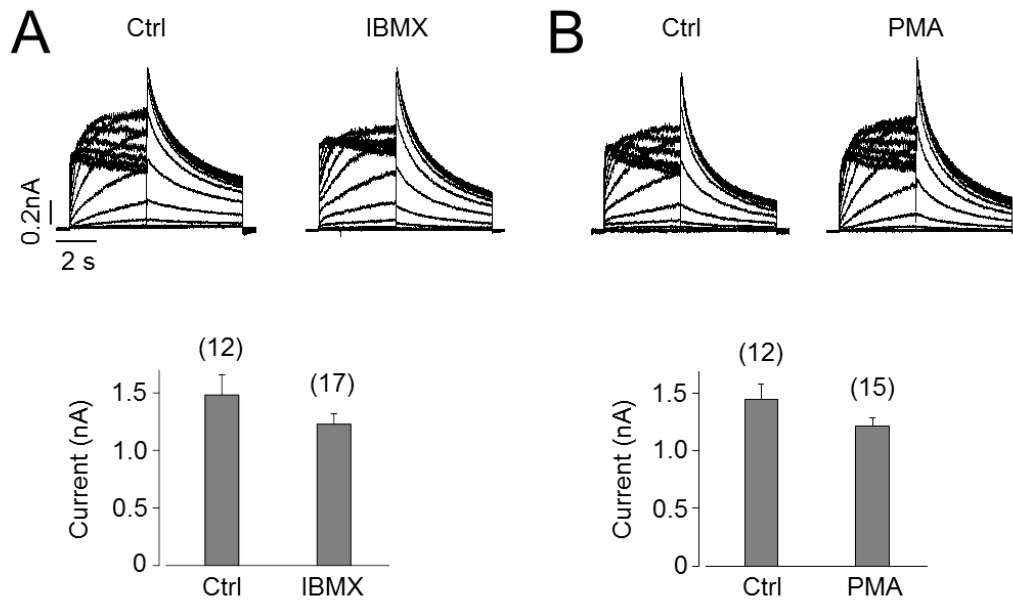


Figure 14: PKA and PKC activations do not alter hERG current (I_{hERG}).

(A) Upper panel: Families of hERG currents without (Ctrl) or with 24-h PKA activation (200 μ M IBMX). Lower panel: The summarized hERG tail current amplitudes under each condition.

(B) Upper panel: Families of hERG currents without (Ctrl) or with 24-h PKC activation (10 nM PMA). Lower panel: The summarized hERG tail current amplitudes under each condition. The numbers above bars indicate the numbers of cells tested from at least three independent experiments.

kDa hERG is on the cell membrane, we treated cells with proteinase K (PK, 200 $\mu\text{g}/\text{ml}$ for 20 min), which 24 h after incubating hERG-HEK cells with PKA (200 μM IBMX) or PKC (10 nM PMA) activator. As shown in Figure 15, PK treatment eliminated the 155-kDa hERG band in WT Ctrl cells and resulted in a degradation fragment with a molecular mass of 65 kDa. Figure 15A shows that PK treatment also eliminated the 155-kDa hERG band in IBMX-treated cell, and as expected, the 65-kDa degradation product after PK digestion was increased. In comparison, while majority of the 155-kDa proteins was cleaved in PMA-treated cells, a small portion was also resistant to PK digestion, suggesting their intracellular localization (Figure 15B). The increased 65-kDa fragments confirm that at least some of the increased 155-kDa hERG proteins are on the plasma membrane, accessible to PK.

2.4 hERG current elevates when PKA or PKC activators were removed after 24-h incubation

The PK experiment in Figure 15 verifies that a portion of hERG proteins increased by PKA and PKC activations are on the membrane while some are inside the cells. Due to the lack of phosphorylated hERG antibody and mutants at the hERG phosphorylation sites, I indirectly investigated the possibility that the increased hERG channels are inhibited by phosphorylation. Since protein dephosphorylation (Misonou *et al.*, 2004) occurs much faster than hERG protein degradation (Ficker *et al.*, 2003), I investigated the effect of removing the phosphorylation stress on hERG current. Twenty-four hours after incubating cells with PKA or PKC activator, I removed those activators and incubated cells in normal MEM for 2, 4, and 6 h. Due to abundantly presented phosphatases in the cell (Heijman *et al.*, 2013), any phosphorylation on hERG protein should be removed when the kinases are no longer presented if they are indeed phosphorylated during PKA and PKC activations. Figure 16 supports the hypothesis that extra hERG channels are inactivated by phosphorylation since the tail current increases gradually with

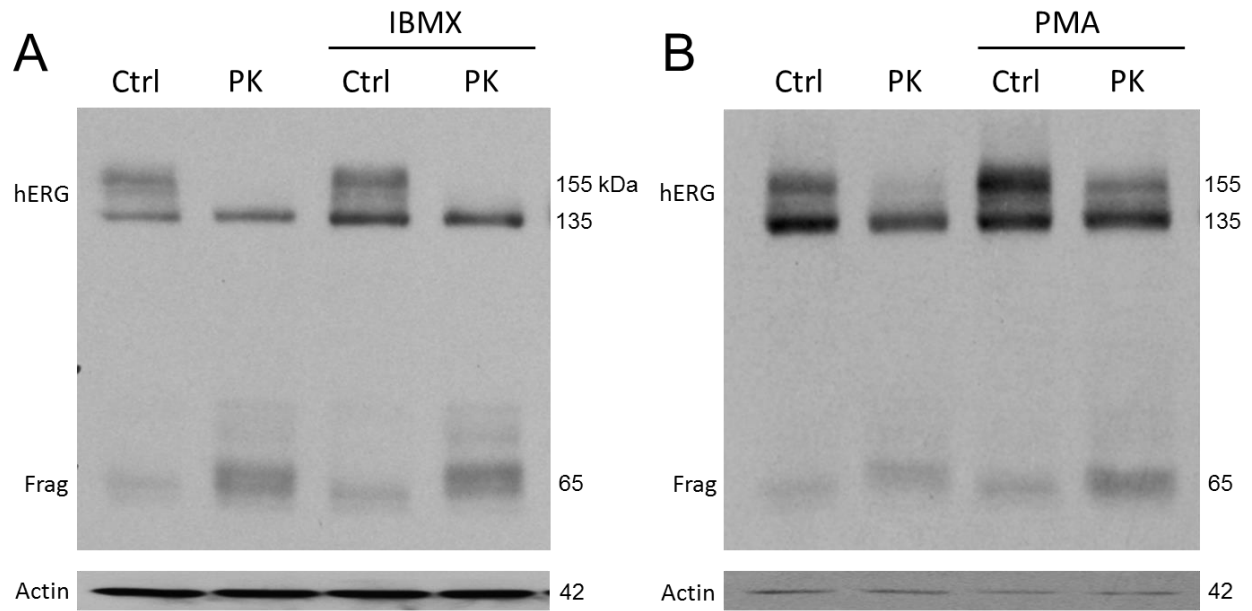


Figure 15: A portion of the increased hERG proteins by PKA and PKC activations are on the plasma membrane.

The PKA- and PKC-mediated hERG protein increase can be digested by proteinase K. hERG-HEK cells were treated with PKA (200 μ M IBMX, panel A) or PKC (10 nM PMA, panel B) activator. Twenty-four hours after PKA or PKC activation, surface proteins were cleaved by proteinase K (PK, 200 μ g/ml for 20 min). Whole-cell lysates were analyzed using Western blot. The digestion fragments appeared after PK cleavages are indicated (Frag).

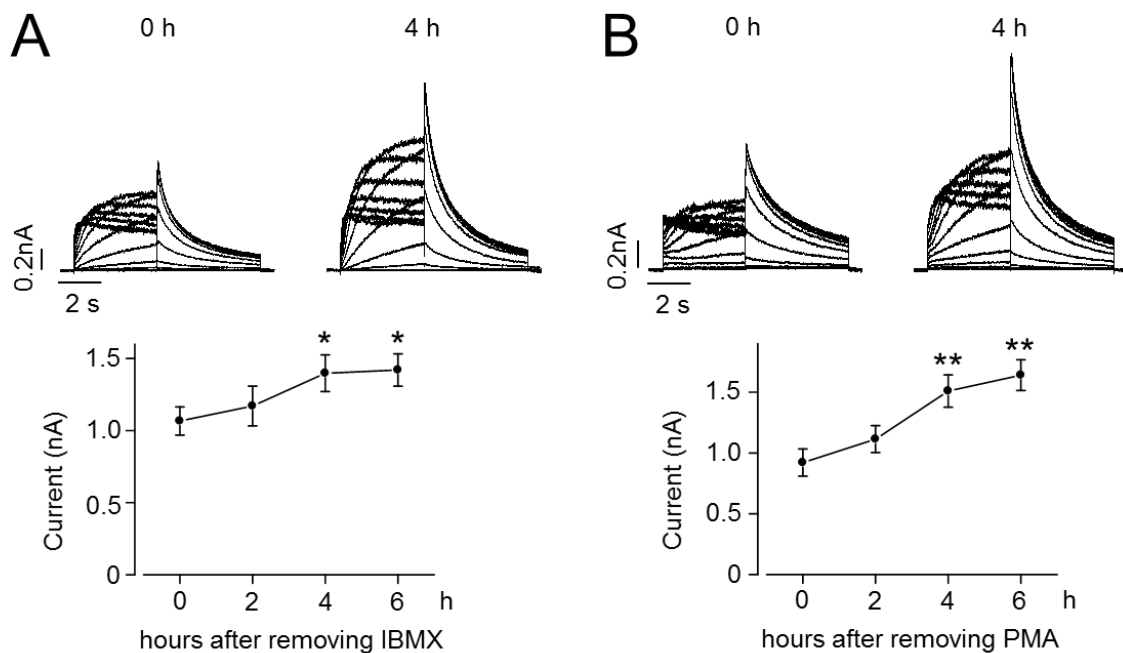


Figure 16: I_{hERG} increases gradually after removing PKA and PKC activators following 24-h PKA or PKC activation.

Families of hERG currents recorded immediately (0 h), and 4 h after 24-h PKA activation by 200 μ M IBMX (A) or PKC activation by 10 nM PMA (B). The tail current amplitudes at 50-mV depolarization voltage recorded immediately (0 h), 2, 4, and 6 h after removing IBMX or PMA activator were summarized underneath representative traces. Error bars, S.E. * $P < 0.05$, ** $P < 0.01$ vs. I_{hERG} at 0 h. n=11-22 from at least three independent experiments.

time upon reincubation after removing extra PKA (Figure 16A) or PKC (Figure 16B) signalling.

2.5 PKC-mediated decrease of hERG channel degradation involves phosphorylation of Nedd4-2

hERG protein degradation is regulated by the ubiquitin system in which the small molecule Ub serves as a tag to target protein for lysosomal degradation (Ciechanover, 2005). The tagging process of hERG protein is mediated by the E3 ubiquitin ligase Nedd4-2 (Guo *et al.*, 2012), and the phosphorylation of Nedd4-2 diminishes its function (Debonneville *et al.*, 2001). Since I have shown previously that muscarinic receptor activation increased hERG expression and function via activation of PKC, which phosphorylates and inactivates Nedd4-2 (Wang *et al.*, 2014), we investigated if PKA activation also results in Nedd4-2 phosphorylation. Figure 17A and B illustrates that cells treated with PKC but not PKA activator showed significantly enhanced ratio of p-Nedd4-2 to Nedd4-2 ($P < 0.05$, $n = 3$ or 4), indicating only PKC impairs Nedd4-2 activity.

Therefore, PKC- but not PKA-mediated enhancement of hERG expression appears to involve impaired hERG channel degradation through inactivation of Nedd4-2. PKA activation did not alter the expression level of either Nedd4-2 or p-Nedd4-2, which further suggests that PKA-mediated enhancement of hERG channel expression does not involve the alternation of Nedd4-2-mediated hERG protein degradation.

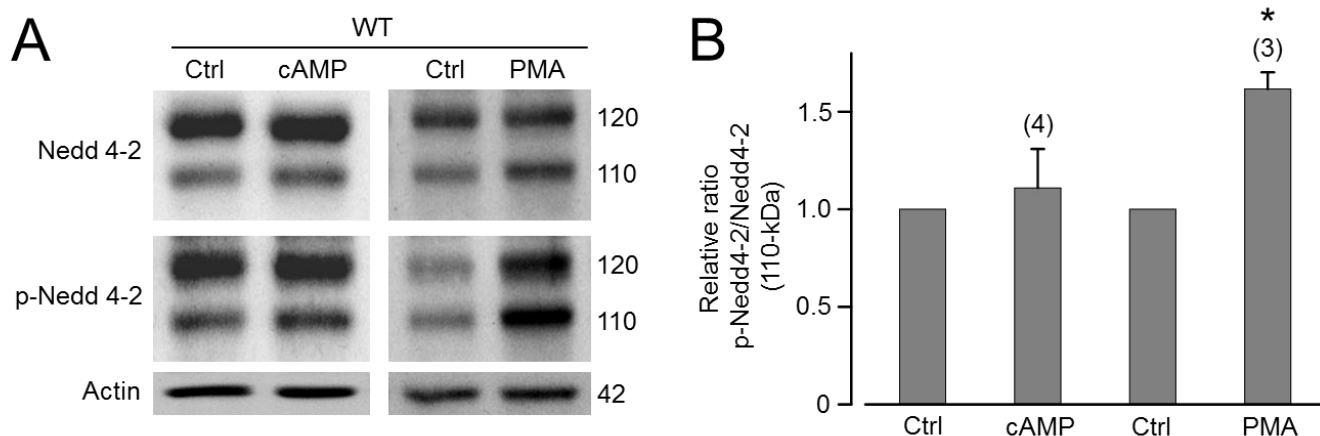


Figure 17: PKC but not PKA impairs hERG protein degradation by phosphorylating Nedd4-2.

(A) Western blot analysis showing total level of Nedd4-2 and phosphorylated Nedd4-2 (p-Nedd4-2) expression under control (Ctrl) condition, after 24-h of PKA (50 μ M CPT-cAMP), or PKC (10 nM PMA) activation. (B) The ratio of p-Nedd4-2 to Nedd4-2 (110-kDa band) treated with CPT-cAMP or PMA compared to the corresponding control (Ctrl) of each treatment. Only the lower 110-kDa bands of Nedd4-2 were examined since we have shown that the 110-kDa band is the form that interacts with hERG protein (Guo *et al.*, 2012). Actin expression was used as a loading control. Error bars, S.E. * $P < 0.05$. n=3 or 4.

Chapter 4: Discussion

The rapidly activating delayed rectifier K⁺ channel encoded by *hERG* is important for the repolarization of cardiac action potentials (Sanguinetti *et al.*, 1995; Trudeau *et al.*, 1995). Loss-of-function mutations in *hERG* or drug blockade decrease I_{hERG} and thus can lead to LQTS (Keating & Sanguinetti, 2001). Therefore, understanding hERG channel regulation is essential for developing strategies to treat and prevent LQTS.

We have previously demonstrated that the overexpression of Nedd4-2 eliminates I_{hERG} (Guo *et al.*, 2012). In the present study, I show that decreasing endogenous Nedd4-2 expression with siRNA transfection increases the current as well as the 155-kDa protein expression of WT hERG channels but not the Nedd4-2-interaction-deficient mutant hERG Y1078A channels. These data indicate that endogenous Nedd4-2 constitutively mediates hERG channel degradation. The fact that Nedd4-2 only targets the 155-kDa hERG proteins is unexpected since both the 135-kDa and the 155-kDa forms contain the PY motif. Our data show that the selectivity of Nedd4-2 in targeting the 155-kDa hERG protein is achieved at least in part by Ndfips.

As a critical molecule in regulating membrane expressed proteins, the activity of Nedd4-2 is constantly regulated. Nedd4-2 possesses a PY motif (LPXY) in its catalytic HECT domain (Staub & Rotin, 2006). In the absence of target proteins, the WW domain of Nedd4-2 binds to its own PY motif in the catalytic HECT domain, retaining Nedd4-2 in its inactive state. Upon activation, Nedd4-2 undergoes conformational changes, shifting the WW domain to expose the HECT domain, which then catalyzes the transportation of Ub from the E2 Ub conjugating enzyme to the substrate (Ingham *et al.*, 2004).

Nedd4 family interacting proteins Ndfip1 and Ndfip2 interact with the all the WW domains of Nedd4 family proteins to assist in their activation process, with the stronger interactions

between PY2 and WW2 as well as WW3 (Harvey *et al.*, 2002; Konstas *et al.*, 2002; Cristillo *et al.*, 2003). The PY motifs of Ndfips confer their ability of binding to Nedd4-2 (Mund & Pelham, 2009). Our data show that both Ndfip1 and Ndfip2 interact with Nedd4-2 as well as hERG protein, and overexpressing Ndfip1 or Ndfip2 significantly reduced WT I_{hERG} . Since both hERG protein and Ndfips possess PY motifs that are recognized by Nedd4-2, there are at least some 3-component complexes formed by hERG channels, Nedd4-2 and Ndfip1 or Ndfip2, as suggested by our co-IP analysis. Moreover, the Ndfip1- and Ndfip2-mediated reduction in I_{hERG} are absent in $\Delta 1073$ and Y1078A hERG channel mutants which have disrupted Nedd4-2 recognition sites. These data suggest that both Ndfip1 and Ndfip2 act through Nedd4-2 to decrease I_{hERG} and cell-surface hERG channel expression.

Ndfips function as regulatory proteins of multiple Nedd4 family ligases, and are expected to affect Nedd4 substrates through various mechanisms (Mund & Pelham, 2009). My results indicate that Ndfip1 is primarily located in the Golgi apparatus, whereas Ndfip2 is present in the MVBs and the Golgi apparatus. These findings are consistent with previous reports concerning the cellular localizations of Ndfip1 and Ndfip2 (Konstas *et al.*, 2002; Harvey *et al.*, 2002; Shearwin-Whyatt *et al.*, 2004). The Golgi apparatus is a critical organelle for hERG protein maturation and complex glycosylation (where the core-glycosylated 135-kDa hERG protein becomes the fully-glycosylated 155-kDa form) (Gong *et al.*, 2002). Thus, recruitment of Nedd4-2 by Ndfip1 and Ndfip2 to the Golgi apparatus may enhance the Nedd4-2-hERG channel interaction. Augmented Nedd4-2 at the Golgi apparatus causes the 155-kDa hERG proteins at the Golgi apparatus being rerouted to the MVBs for lysosomal degradation instead of being trafficked to the plasma membrane. Therefore, the mature (155-kDa) hERG channel expression

was reduced. This notion is consistent with our data showing that the matured 155-kDa form of hERG proteins from the 135-kDa form is significantly reduced by both Ndfip1 and Ndfip2.

Ndfip1 and Ndfip2 share only 50% amino acid sequence homology, and thus may play distinct roles in the regulation of Nedd4-2 activities (Cristillo *et al.*, 2003). While both Ndfip1 and Ndfip2 decreased I_{hERG} and surface-expressed hERG protein expression, only Ndfip1 decreased the 155-kDa hERG band in whole-cell protein. Ndfip-2-transfected cells displayed reduced I_{hERG} and decreased 155-kDa band expression in cell-surface proteins. However, Ndfip2 overexpression did not change the 155-kDa hERG expression in whole-cell protein. These data may imply an accumulation of the internalized 155-kDa hERG channels in these cells. This notion is consistent with my confocal images showing an intracellular punctate staining of hERG proteins in Ndfip2-transfected WT hERG-HEK cells. The mechanisms underlying the Ndfip2-induced changes in hERG channel function and protein expression are not entirely clear. However, my data show that Ndfip2 is primarily located in the MVBs, which is consistent with previous reports (Konstas *et al.*, 2002; Harvey *et al.*, 2002; Shearwin-Whyatt *et al.*, 2004). The overexpression of Ndfip2 and the subsequent recruitment of Nedd4-2 may be associated with degradation of certain molecules that are important for MVB function, leading to impaired degradation (Saksena *et al.*, 2007). Antibodies targeting the vacuolar protein sorting-associated protein 28 (Vps28) in endosome have been shown to impair the degradation of epithelial growth factor, and cause intracellular accumulation of the ubiquitinated substrates (Bishop *et al.*, 2002). There is another way that the function of MVBs could be impaired, which is that as a structural protein of the MVBs, the overpopulated Ndfip2 disrupts the structure of MVBs, resulting in its impaired function. Since MVBs are involved in the degradation of internalized hERG channels

(Sun *et al.*, 2011), decreased MVB function would result in an accumulation of internalized mature hERG channels.

While the first half of this work discussed how the specificity of Nedd4-2 toward the 155-kDa band is achieved, the second half will consider the ways to enhance hERG expression and function by targeting Nedd4-2.

The incidence of unexplained abnormal cardiac events increases dramatically with adrenergic activation (Yasutake & Avkiran, 1995) as a result of augmented sympathetic stimulation through elevated catecholamine release under conditions such as emotional stress and intense exercise. In the present work, we investigated the activation of downstream pathways of both α and β adrenergic activation (PKC and PKA pathway, respectively) on the protein expression and functionality of hERG potassium channels, which are responsible for cardiac repolarization.

I found that although both PKC and PKA activations upregulate hERG protein expression (Figure 13) but through different pathways. The fact that upon PKA activation (downstream of β adrenergic stimulation) both the core-glycosylated 135-kDa band and the fully-glycosylated 155-kDa band are increased suggest that PKA-mediated enhancement of hERG protein may be through enhanced protein synthesis. This idea is supported by the findings that increased hERG protein colocalizes with the ER marker, calnexin (Chen *et al.*, 2009). One of the main substrates of PKA phosphorylation, a transcription factor called the cAMP response element binding protein, may be involved in the augmented hERG protein expression (Delghandi *et al.*, 2005). In addition, the fact that the proportion of inactivated (phosphorylated) Nedd4-2 (ratio of p-Nedd4-2/Nedd4-2) is not altered with PKA activation further confirms that degradation, at least the

main pathway through Nedd4-2, is not involved in PKA-mediated enhancement of hERG protein expression.

Since the activation of β adrenergic receptor is linked to both Gs (stimulatory) and Gi (inhibitory) protein, the increased hERG expression and function as a result of β adrenergic stimulation may be associated with Gs protein whereas the decreased hERG expression is linked to Gi protein, which acts to inhibit the function of adenylyl cyclase to reduce cAMP production and thus decreases PKA signalling. This might explain the discrepancy of the opposite effects of β adrenergic stimulation on hERG channel expression and function. Furthermore, the use of β adrenergic receptor agonist in treating Torsades de Pointes (Chao *et al.*, 1996) may aim to enhance the activation of Gi protein, and thus reduce PKA signalling. However, the factors that determine the activation of Gs or Gi protein, but not the other, upon β adrenergic activation require further research.

PKC is the downstream signalling pathway of α adrenergic activation. PKC activator (PMA)-treated cells also showed enhanced hERG protein expression. However, in comparison to those treated with PKA activators, the 135-kDa core-glycosylated hERG band did not increase as much as those activated by PKA. I have shown that PKC enhances hERG expression mainly through decreasing the degradation rate of hERG protein. The finding that the proportion of phosphorylated (inactive) Nedd4-2, the E3 Ub ligase mediating hERG channel degradation, is significantly increased further confirms the theory that PKC activation impairs hERG protein degradation cascade and results in the augmented level of 155-kDa band.

Surprisingly, the functionality of hERG channels remained unchanged, which did not reflect the enhanced 155-kDa protein expression after PKA or PKC activation. Two possible explanations may account for the lack of hERG current enhancement, despite an elevated hERG

protein expression upon PKA and PKC activation. One is that most of the newly synthesized hERG proteins have not yet reached the plasma membrane. However, PK cut shows the cleavage of most of the 155-kDa hERG protein and with increased degradation fragment after PKA and PKC activation. These observations led me to hypothesize that the increased hERG channels on the cell surface may be inactivated and not functional. Since hERG channels can be inactivated by phosphorylation, and due to the presence of PKA and PKC, I rationalize that the extra hERG proteins might be turned off (Thomas *et al.*, 1999; Thomas *et al.*, 2003). Since cardiac electrophysiology have been shown to be constitutively regulated by kinases and phosphatases (Heijman *et al.*, 2013) we removed PKA or PKC activators after 24-h incubation, and therefore release the phosphorylation (inactivation) stress. In fact, I saw an increase in hERG current 2 h after removing PKA and PKC activators. The increase in current reached a plateau 4 h after removing the PKA activator. The fact that the current did not increase as much as the protein did suggests: 1. some channels remain phosphorylated and inactivated; 2. not all augmented hERG proteins are inserted into the membrane. The remaining 155-kDa hERG protein upon PKC activation after PK digestion also suggests that some 155-kDa proteins are not inserted into the plasma membrane. Therefore, I reason that there are certain molecules associated with the insertion and anchoring of hERG protein to plasma membrane that might not be upregulated by PKA or PKC activation. The A kinase anchoring protein (AKAP) might be involved in stabilization of hERG on plasma membrane (Li *et al.*, 2008). AKAP9 has already been shown to be responsible for modulating signalling cascade in KCNQ1/KCNE1 complex (Marx & Kurokawa, 2006), which conducts the slowly activating delayed rectifier current I_{Ks} , and is also critical for the repolarization of the cardiac action potential.

Inconsistent with my findings, many research groups found decreased hERG current upon activation of PKC (Bian *et al.*, 2001; Cockerill *et al.*, 2007). The PKC activation via M3-muscarinic receptor activation by methacholine reduced the hERG current in HEK293 cells (Cockerill *et al.*, 2007). Native I_{Kr} from the guinea pig cardiomyocytes was reduced upon activation of PKC by DAG analog phorbol esters. However, the activation of PKC through M3-muscarinic receptor not only has an effect on PKC signalling but also induces intracellular Ca^{2+} release (Kamp & Hell, 2000), which has been shown to cause a reduction in hERG current (Ficker *et al.*, 2004).

There also seems to be a difference in the long term and short term effects of PKA and PKC activations on hERG current, suggesting their acute inhibition of the channel current and long term enhancement of hERG proteins. The short-term effect (minutes) (Cui *et al.*, 2000; Thomas *et al.*, 2003; Thomas *et al.*, 2004; Wang *et al.*, 2009) seems to be inhibitory, whereas long term (24 h to 72 h) (Chen *et al.*, 2009; Chen *et al.*, 2010; Krishnan *et al.*, 2012; Sroubek & McDonald, 2011) incubation with PKA or PKC activators enhances hERG protein expression and thus the current.

The discrepancy regarding the effect of PKC activation on hERG current or native I_{Kr} can also lie on the distinct effects exerted by multiple PKC isoforms. The evidence that PKC δ is associated with cardiac ischemia (Chen *et al.*, 2001), and studies showing reduced hERG expression in hERG-HEK cells under hypoxia (Nanduri *et al.*, 2009) raises the possibility of PKC δ regulating hERG expression during hypoxia.

I have demonstrated that the 24-h activation of both PKA and PKC pathways enhances hERG protein expression while inhibiting channel function via phosphorylation; therefore, no enhancement in channel function can be observed immediately after 24-h PKA or PKC

activation. It is only when the phosphorylation stress is released by phosphatases can the increase in hERG current be revealed. Moreover, PKA and PKC exert their effect on hERG protein expression through different pathways. PKA enhances hERG protein synthesis whereas PKC slows down hERG degradation by inactivating Nedd4-2 via phosphorylation.

A speculative scheme illustrating the distinct effects of Ndfip1, Ndfip2, PKA and PKC on hERG expression is shown in Figure 18. hERG transcription may be positively regulated by PKA activation at the nucleus. After being synthesized in the ER and fully glycosylated, hERG channels (155-kDa) in the Golgi apparatus can be targeted by Nedd4-2 which is recruited and activated by Ndfip1 and Ndfip2 (to a lesser extent). Nedd4-2 ubiquitinates and directs the 155-kDa hERG protein presented in the Golgi apparatus to be degraded by MVBs-lysosomal pathway. As a result, a reduced amount of newly synthesized hERG proteins are transported to the plasma membrane, leading to a lower level of surface-expressed hERG channels. However, the exact pathway of how hERG is transferred from Golgi apparatus to MVBs is not clear. On the other hand, overexpressed Ndfip2 recruits and activates Nedd4-2 at the MVBs, resulting in delayed hERG protein degradation via decreased MVBs function, which causes an accumulation of internalized hERG channels (Piper & Katzmann, 2007). The ability of Nedd4-2 in ubiquitinating hERG proteins can be impaired by PKC-mediated phosphorylation. The effects of Ndfip1 and Ndfip2 overexpression on the Golgi apparatus and MVBs, as well as PKC-mediated Nedd4-2 phosphorylation provide novel insights into Nedd4-2 function in cell biology, and may have implications for the trafficking of other cellular molecules.

In summary, my present study demonstrates that various factors can regulate hERG channels via targeting Nedd4-2 which mediates hERG protein degradation. I show that Ndfip1 and Ndfip2 interact with Nedd4-2 via distinct pathways to control hERG channel expression in the plasma

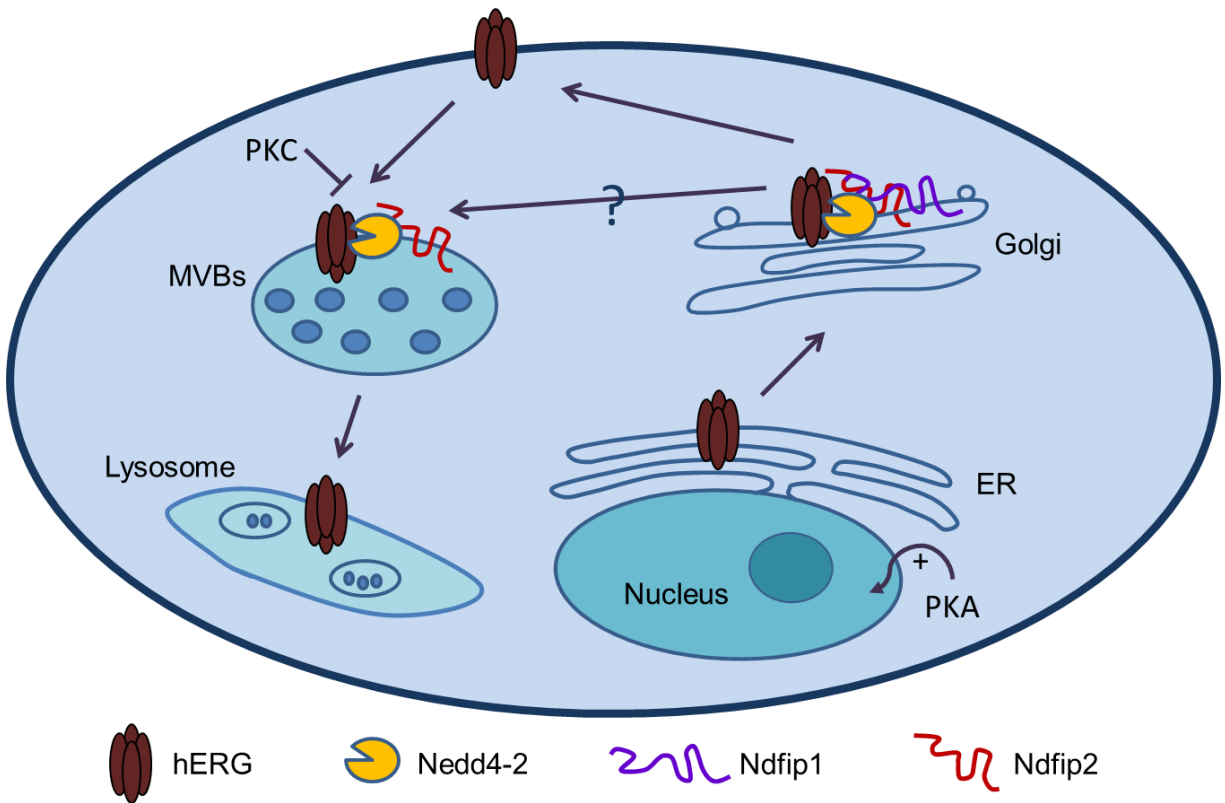


Figure 18: A schematic illustration of Ndfip1-, Ndfip2-, PKA-, and PKC-mediated regulation of hERG channels.

PKA activation may positively regulate hERG protein expression at the transcription level in the nucleus (arrow with “+” sign). Ndfip1 (and Ndfip2 to a lesser extent) reduces mature hERG protein expression at the Golgi apparatus while Ndfip2 decreases MVB-mediated degradation of internalized hERG channels. Golgi apparatus-localized Ndfip1 (and Ndfip2 to a lesser extent) recruits and activates Nedd4-2 to degrade hERG protein through MVBs-lysosomal pathway during channel maturation. Ndfip2 recruits and activates Nedd4-2 in MVBs to interfere with the MVBs-mediated hERG protein degradation, leading to an accumulation of internalized mature hERG channels inside the cells. Furthermore, PKC, acting on the degradation pathway of hERG channels, phosphorylates Nedd4-2 to inhibit hERG channel ubiquitination, and thus degradation.

membrane and thus its function. In addition, PKA and PKC signalling constitutively regulate both hERG expression and function. Our findings provide further information concerning the regulation of hERG channels, which is useful for understanding impaired current and developing new strategies to enhance hERG channel trafficking. Furthermore, any molecules involved in such signalling cascades can be possible target to prevent the arrhythmia induced by adrenergic stimulation.

Future Directions

This current work demonstrates that Ndfip1 and Ndfip2 facilitate Nedd4-2 in specifically targeting the 155-kDa hERG protein in hERG-HEK stable cell line, and the overexpression of Ndfip1 and Ndfip2 decreases I_{Kr} in neonatal cardiomyocytes. It would be interesting to investigate the link between Ndfip mutations and cardiac electrophysiological abnormalities. It would also be useful to screen for any drugs targeting Ndfips, which might provide insights in unexplained cardiac arrhythmia. Furthermore, we demonstrated that PKA and PKC regulate hERG channels via altering expression and phosphorylation. Our study raises the possibility that channel phosphorylation is a self-regulatory mechanism to prevent abnormally large hERG current as a result of enhanced protein expression to maintain the current homeostasis.

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