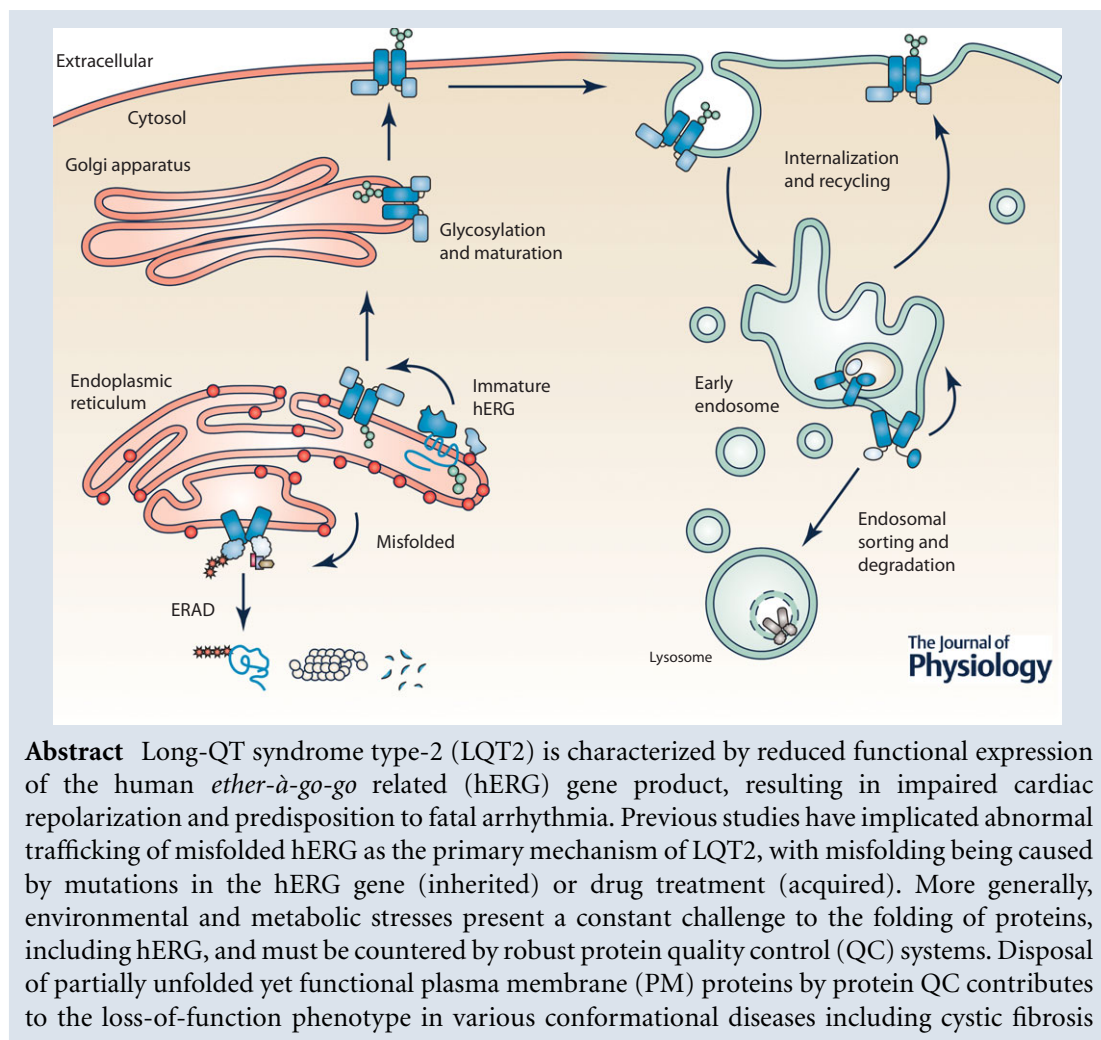


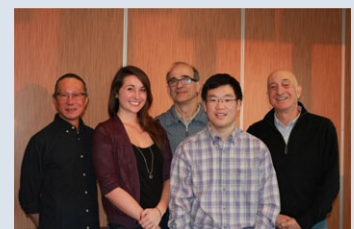
SYMPOSIUM REVIEW

hERG quality control and the long QT syndrome

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Abstract Long-QT syndrome type-2 (LQT2) is characterized by reduced functional expression of the human *ether-à-go-go* related (hERG) gene product, resulting in impaired cardiac repolarization and predisposition to fatal arrhythmia. Previous studies have implicated abnormal trafficking of misfolded hERG as the primary mechanism of LQT2, with misfolding being caused by mutations in the hERG gene (inherited) or drug treatment (acquired). More generally, environmental and metabolic stresses present a constant challenge to the folding of proteins, including hERG, and must be countered by robust protein quality control (QC) systems. Disposal of partially unfolded yet functional plasma membrane (PM) proteins by protein QC contributes to the loss-of-function phenotype in various conformational diseases including cystic fibrosis

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(CF) and long-QT syndrome type-2 (LQT2). The prevalent view has been that the loss of PM expression of hERG is attributed to biosynthetic block by endoplasmic reticulum (ER) QC pathways. However, there is a growing appreciation for protein QC pathways acting at post-ER cellular compartments, which may contribute to conformational disease pathogenesis. This article will provide a background on the structure and cellular trafficking of hERG as well as inherited and acquired LQT2. We will review previous work on hERG ER QC and introduce the more novel view that there is a significant peripheral QC at the PM and peripheral cellular compartments. Particular attention is drawn to the unique role of the peripheral QC system in acquired LQT2. Understanding the QC process and players may provide targets for therapeutic intervention in dealing with LQT2.

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Abstract figure legend hERG cellular processing and quality control. Nascent hERG channels are inserted into the endoplasmic reticulum membrane where they undergo N-linked glycosylation. Properly folded hERG is exported to the Golgi apparatus where there is additional glycosylation prior to export to the plasma membrane (PM). Nascent hERG channels that fail to fold properly are targeted for Endoplasmic Reticulum Associated Degradation (ERAD). PM resident hERG channels are internalized and either cycled back to the PM or, if misfolded, are targeted for lysosomal degradation.

Abbreviations CF, cystic fibrosis (CF); CFTR, cystic fibrosis transmembrane conductance regulator; CHIP, C-terminus of Hsc70-interacting protein; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; hERG, Human *ether-à-go-go* related gene; HSP, heat shock protein; LQT2, long-QT syndrome type-2; NEF, nucleotide exchange factor; PM, plasma membrane; QC, quality control; WT, wild-type.

Introduction

The *human ether-a-go-go related gene* (*hERG1*, gene name *KCNH2*) encodes the Kv11.1 inwardly rectifying K⁺ channel commonly referred to as hERG. In cardiac myocytes hERG is expressed as the rapidly activating delayed rectifier potassium current (I_{Kr}) that is involved in cardiac repolarization (Trudeau *et al.* 1995). Loss of hERG function is associated with long-QT syndrome type-2 (LQT2), characterized by impaired ventricular repolarization, extended action potential duration and increased risk of potentially fatal *torsades de pointes* arrhythmia (Keating & Sanguinetti, 2001; Sanguinetti & Tristani-Firouzi, 2006). The loss of hERG function as seen in LQT2 can be caused by mutations in the hERG gene (inherited LQT2) or as a result of off-target drug effects (acquired LQT2). As with other proteins, the normal maturation and function of the hERG channel requires proper protein folding and conformational integrity, which is constantly challenged by physical, environmental and metabolic stresses. Consequently, cells require robust protein quality control (QC) systems to monitor folding status and to direct unfolded molecules within each cellular compartment to either refolding or degrading pathways. Owing to the fact that a growing number of diseases are related to protein misfolding, the study of protein QC mechanisms as potential therapeutic targets has received increasing attention. A large subset of acquired and inherited LQT2 involves degradation of

non-native hERG channels by the protein QC machinery, resulting in a reduction in cell-surface expression and contributing to the overall loss of functional phenotype. In many cases, misfolded channels targeted for degradation are at least partially functional, making protein QC systems a significant player in LQT2 pathogenesis (Anderson *et al.* 2006; Ke *et al.* 2013). For many years, the hERG loss-of-expression phenotype was attributed to impaired biosynthetic maturation and plasma membrane (PM) insertion. Recent work has uncovered an extensive QC network targeting misfolded plasma membrane proteins, including hERG, for degradation. Removal of misfolded hERG from the cell periphery is likely to act in parallel with the established biosynthetic maturation defect and almost certainly plays a significant role in LQT2 pathogenesis. This review will highlight recent progress on the mechanisms underlying hERG quality control and their contribution to LQT2 pathogenesis.

Background

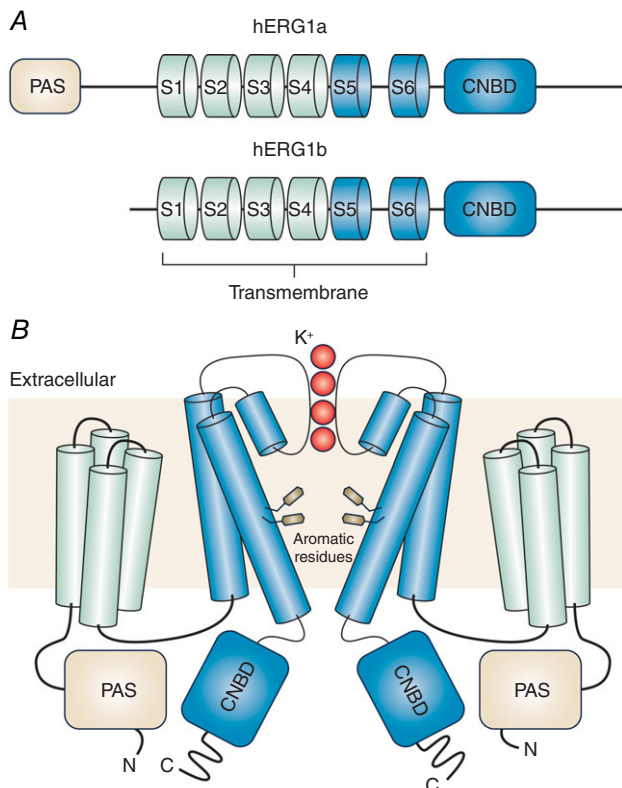
Structure and maturation of hERG. The hERG channel is a tetrameric (Vandenberg *et al.* 2012) structure containing a transmembrane core and two cytosolic structures: an N-terminal Per-Arnt-Sim (PAS) domain and a C-terminal cyclic nucleotide-binding domain (CNBD) (Fig. 1A). The transmembrane region forms the voltage sensor, the selectivity filter and the ion-conducting pore. Interactions between the hERG cytosolic domains and the

transmembrane region regulate channel-gating kinetics (Vandenberg *et al.* 2004).

In cardiac cells, hERG is expressed as two isoforms: hERG1a, which constitutes the full-length channel that conducts a current at the cell surface, and hERG1b, which lacks most of the cytosolic N-terminus including the PAS domain and instead has a short 35-residue N-terminal cytosolic sequence (Phartiyal *et al.* 2008) (Fig. 1A). Both hERG1a and -1b homotetramers form functional ion channels; however, whilst hERG1a is expressed at the cell surface, hERG1b is confined to the ER in the absence of hERG1a co-expression, presumably due to the presence of an 'RXR' ER retention signal on the hERG1b N-terminus (Phartiyal *et al.* 2008). Consequently, most studies investigating hERG cellular processing and quality control rely upon exogenous overexpression of hERG1a

alone. In cardiac myocytes, incorporation of hERG1b regulates the kinetics of channel gating and is essential for normal cardiac repolarization (Larsen *et al.* 2008; Jones *et al.* 2014); whether the addition of hERG1b subunits influences hERG folding/misfolding remains relatively unexplored.

As with other polytopic plasma membrane proteins, nascent hERG channels are synthesized and N-linked glycosylated in the endoplasmic reticulum (ER) (Fig. 2). Channels are then trafficked to the Golgi and subjected to additional glycosylation before being exported to the plasma membrane (PM) (Zhou *et al.* 1998; Petrecca *et al.* 1999; Gong *et al.* 2002). This additional glycosylation increases the apparent molecular mass from 135 kDa (core-glycosylated form) to 155 kDa (mature complex glycosylated form) (Fig. 2 inset). At the cell periphery, mature hERG channels cycle between the PM and endocytic compartments (Apaja *et al.* 2013).



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Figure 1. hERG channel structure

A, domain structure of hERG1a (top) and hERG1b (bottom). hERG1a contains a cytosolic Per-Arnt-Sim (PAS) and cyclic nucleotide binding domain (CNBD), as well as 6 transmembrane helices S1–S6. The hERG1b N-terminus lacks a PAS domain and instead contains a unique 35-amino-acid sequence. Domains are not drawn to scale. **B**, putative topology of the hERG K⁺ channel tetramer. For clarity, only one pair of opposing subunits comprising the hERG tetramer are shown. K⁺ ions passing through the channel selectivity filter are shown in red. Also shown are aromatic amino acids Y652 and F656 involved in drug-induced hERG block.

Inherited LQT2. The congenital form of LQT2 is dominantly inherited, with a prevalence estimated to be 1:6000. It is linked to nearly 500 hERG mutations, with no single mutation being predominant (Schwartz *et al.* 2009). It is noteworthy that over 30% of these LQT2 mutations can cause nonsense mutations, resulting in a truncated, non-functional form of hERG mRNA that is likely to result in nonsense mediated mRNA decay (Gong *et al.* 2007). Studies have shown that deletion/truncation at the hERG CNBD is poorly tolerated (Akhavan *et al.* 2005). However, mRNA truncated downstream of the CNBD can produce functional channels if nonsense-mediated mRNA decay is inhibited (Gong *et al.* 2011). The dominant mechanisms for inherited LQT2 pathogenesis identified to date are the presence of missense mutations in hERG, which result in altered channel function and reduced channel expression due to impaired ER exit and accelerated degradation. The latter has been proposed as the predominant mechanism, accounting for almost 90% of the mutants tested (Anderson *et al.* 2006, 2014; Ke *et al.* 2013) (see Fig. 2 for example). There is significant variation in the expression defect between mutations; some completely abolish expression of mature hERG while others exhibit a milder phenotype (Ke *et al.* 2013; Anderson *et al.* 2014). In some cases, rescue of hERG expression by incubation at low temperature restored PM channel activity (Anderson *et al.* 2006, 2014; Ke *et al.* 2013). Since low temperature rescue is not a clinical treatment option, there is more emphasis placed on folding and trafficking pathways that could restore abusive inherited mutations. Though it has not yet been explored, it is also interesting to speculate on the presence of modest hERG mutations that produce a negligible clinical phenotype in isolation, but possibly act synergistically with pharmacological or environmental factors to generate a more severe defect under certain conditions.

Acquired LQT2. The hERG channel is the target for the vast majority of drug-induced arrhythmias and represents a major cause of withdrawal of drugs from the market (Roden, 2004; Waring *et al.* 2015). A recent *in silico* study suggests that up to 5% of approved drugs bind hERG as an off-target effect (Lounkine *et al.* 2012). In general, drugs can impact hERG either directly by binding to the channel or indirectly through modifying cellular processes.

The majority of compounds affecting hERG function block channel conductance by directly binding two non-conserved aromatic amino acid residues exposed within the channel pore (Y652, F656, Fig. 1B) (Lees-Miller *et al.* 2000; Mitcheson *et al.* 2000; Sanchez-Chapula *et al.* 2002; Fernandez *et al.* 2004). A subset of hERG blockers has additional effects on channel trafficking. Some hERG blockers have been shown to reduce hERG PM expression by impairing ER exit and accelerating

degradation from the cell surface (Dennis *et al.* 2011). These effects are likely to synergize with the functional block to produce the acquired LQT2 phenotype. Interestingly, other hERG blockers have the opposite effect on expression and trafficking, being able to increase the expression of mutant hERG channels (Zhou *et al.* 1998; Ficker *et al.* 2002; Kuryshev *et al.* 2005; Rajamani *et al.* 2006; Takemasa *et al.* 2008). The structural basis determining whether a hERG blocker promotes or impairs hERG expression remains unknown. Pharmacological rescue of mutant hERG expression presents a promising therapeutic strategy for inherited LQT2; however, current hERG ‘pharmacochaperones’ are limited by concomitant functional block of the rescued channels. A number of hERG ‘activators’ bind to sites outside the channel pore and modulate hERG gating (Kang *et al.* 2005; Casis *et al.* 2006; Perry *et al.* 2007; Grunnet *et al.* 2011). Whether

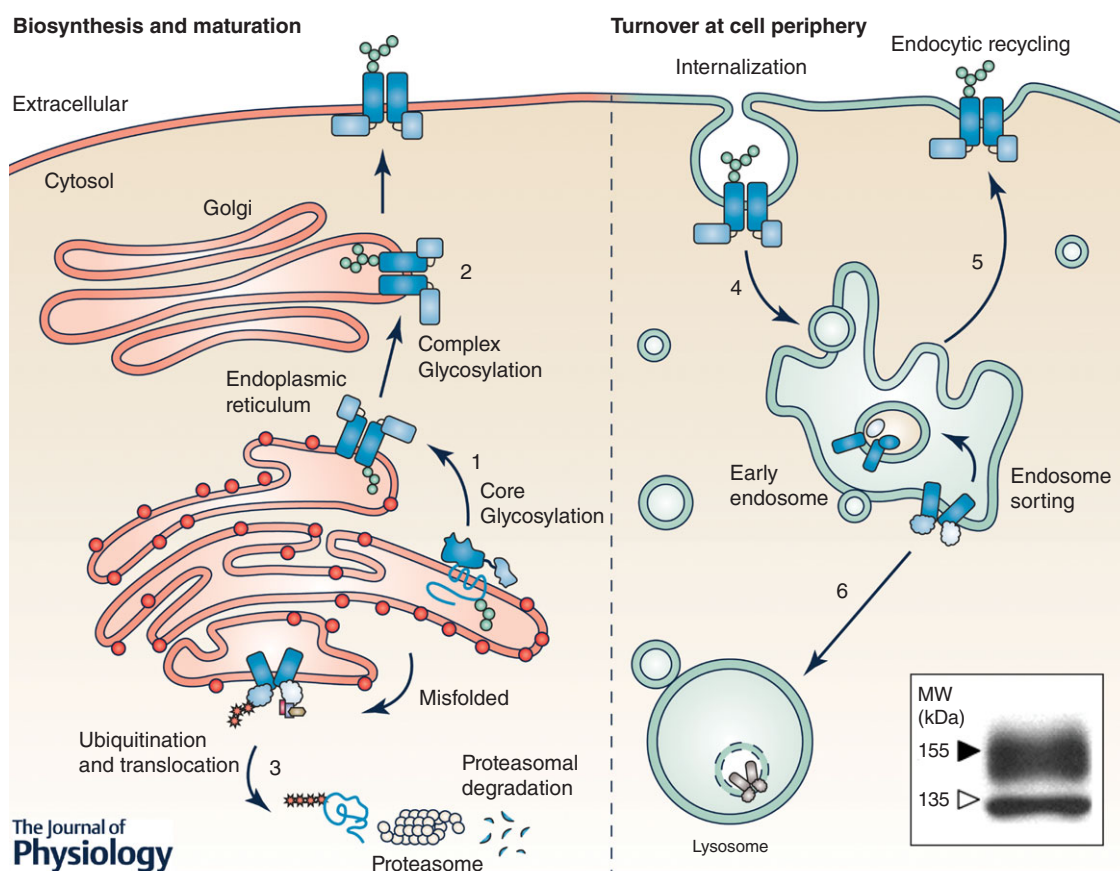


Figure 2. hERG cellular processing

Overview of hERG cellular processing. Nascent channels are cotranslationally inserted into the ER membrane (1) where they undergo N-linked glycosylation to an apparent molecular mass of 135 kDa. Upon proper folding and assembly, hERG is exported to the Golgi where it is subject to additional glycosylation, increasing the apparent molecular mass to 155 kDa prior to export to the plasma membrane (2). Nascent channels, which fail to fold at the ER, are targeted for proteasomal degradation (3). PM-resident hERG is internalized (4) and either recycled back to the PM (5) or targeted for lysosomal degradation (6). Inset, typical immunoblot of WT hERG. Indicated are the immature 135 kDa core-glycosylated and mature 155 kDa complex glycosylated forms (open and filled arrow respectively). hERG is detected in stably transfected HeLa cells by immunoblotting against an engineered HA-epitope in the S1–S2 extracellular loop (Ficker *et al.* 2003).

binding to these non-pore sites can rescue mutant hERG trafficking remains to be established.

Pharmacological agents can also impact hERG indirectly in the absence of binding. This was found to be the case for acquired LQT2 associated with cardiac glycosides and diuretic-induced hypokalaemia. Depletion of intracellular K^+ via cardiac glycosides and depletion of extracellular K^+ have both been shown to destabilize the hERG conformation or defect maturation (Wang *et al.* 2007; Guo *et al.* 2009).

hERG domains. Mutations affecting hERG conformation have been identified in both the PAS and CNBD cytosolic domains, as well as within the membrane-spanning helices and extracellular/intracellular loops (Anderson *et al.* 2006, 2014). Deletion of the entire CNBD or any conserved structural motif within the CNBD has a severe impact on channel expression, suggesting that an intact and properly folded CNBD is necessary for stable channel conformation and trafficking (Akhavan *et al.* 2005). Indeed, mutations in this region tend to be more severe in their trafficking defect, and poorly correctible by low-temperature incubation and/or pharmacochaperones (Anderson *et al.* 2014).

The role of the N-terminal PAS domain in hERG folding is less clear-cut. Truncated hERG1a lacking the entire N-terminal region appears to traffic normally (Phartiyal *et al.* 2008; Ke *et al.* 2014); however, a significant number of LQT2-associated mutations reducing channel expression have been identified in this region (Harley *et al.* 2012). Recent studies have investigated the impact of these mutations on both the thermal stability of the isolated domain and hERG gating kinetics (Harley *et al.* 2012; Ke *et al.* 2013). Most of the mutations examined reduced the thermal stability of the isolated domain. However, in some cases the extent of thermal destabilization did not correspond with the severity of trafficking defect. In many cases, channel destabilization was associated with abnormal gating kinetics, suggesting altered domain–domain interactions. The hERG PAS domain contains a hydrophobic patch that is thought to be involved in domain interactions (Morais Cabral *et al.* 1998). It is possible that such interactions are required to conceal this hydrophobic area, which, if exposed, could be recognized by protein QC systems. Alternatively, it is possible that while complete absence of a PAS domain interaction is tolerated, an abnormal interaction negatively affects the co-operative folding of other domains. The relative importance of local destabilization of the PAS domain *vs.* loss of domain–domain interactions remains to be directly examined.

Acquired LQT2 by either direct drug binding or K^+ depletion is thought to act at the channel pore. In a recent study, we found that perturbations at the channel pore (via K^+ depletion) or by a mutation in an

extracellular loop induced unfolding of the hERG cytosolic domains (Apaja *et al.* 2013). This suggests that local perturbations within the hERG transmembrane region trigger co-operative unfolding of additional domains. A similar mechanism whereby misfolding of one domain triggered co-operative misfolding of others was shown to contribute to the conformational destabilization caused by several disease-associated cystic fibrosis transmembrane conductance regulator (CFTR) mutations, including $\Delta F508$ (Du & Lukacs, 2009; Rabeh *et al.* 2012).

ER quality control

Nascent hERG at the ER must successfully undergo several stages of protein folding prior to Golgi export, including folding of the polypeptide chain, post-translational folding after release from the ribosome, and oligomer assembly. To guide hERG and other proteins through these stages, the ER has its own quality control system (ERQC). There has been much emphasis on understanding the key players of the ERQC and their role in different protein misfolding diseases. Impaired hERG biosynthetic secretion and maturation, presumably due to the action of ERQC machinery, remains one of the most established mechanism for LQT2 pathogenesis (Kuryshv *et al.* 2005; Anderson *et al.* 2006; Rajamani *et al.* 2006; Takemasa *et al.* 2008).

The mediators of the ERQC system are the molecular chaperones. These proteins both promote the proper folding of nascent polypeptides but also send terminally misfolded substrates for degradation. Degradation of misfolded substrates is mediated by a process called ER associated degradation (ERAD), involving binding of molecular chaperones and co-chaperones, recruitment of ubiquitination machinery, dislocation of transmembrane segments from the ER membrane into the cytosol, and proteasomal degradation. We will discuss the ERQC in two parts: the chaperone/co-chaperone system and the ubiquitination/degradation machinery associated with ERAD.

HSP70/90 chaperone systems

The predominant molecular chaperones involved in ERQC are the heat-shock protein (Hsp) families Hsp70 and Hsp90, along with associated co-chaperones (Fig. 3) (Hartl *et al.* 2011). The function of these proteins has been covered in detail elsewhere (Mayer, 2013; Young, 2014). Briefly, Hsp70 cycles between a high substrate affinity (ADP-bound) state and a low substrate affinity (ATP-bound) state. The Hsp40/DNAJ co-chaperone family assists in Hsp70 substrate binding by stimulating the hydrolysis of ATP to ADP. Conversely, nucleotide exchange factors (NEFs) stimulate the release of substrate through the exchange of bound ADP for ATP.

Hsp70-bound substrates can be passed to Hsp90, which further promotes substrate folding (Fig. 3). The Hsp90 chaperone family also functions via an ATPase cycle that is mechanistically unrelated to Hsp70, and is assisted and modulated by a complicated co-chaperone network (Pearl & Prodromou, 2006; Li *et al.* 2012). The Hsp70 and Hsp90 networks have both been implicated in the folding and trafficking mechanisms of hERG (Ficker *et al.* 2003).

The chaperone systems need to strike a balance between refolding of salvageable substrates and degradation of terminally misfolded molecules. The two primary members of the Hsp70 family, the constitutively expressed heat-shock cognate (Hsc)-70 and the stress inducible Hsp70, appear to play opposing roles although they are regulated by the same co-chaperones. Overexpression of Hsp70 increases wild-type (WT)-hERG expression levels and I_{Kr} , whereas Hsc70 overexpression does not (Ficker *et al.* 2003; Li *et al.* 2011). Hsp70 and Hsc70 were previously proposed to play opposite roles in the ER processing of ENaC channels, suggesting that Hsp70 and Hsc70 may promote folding and degradation, respectively (Goldfarb *et al.* 2006). The reason for this difference between Hsp70 and Hsc70 is unknown but may involve the co-chaperone C-terminus of Hsc70-interacting protein (CHIP), an E3 ubiquitin ligase (see below). Furthermore, trafficking-deficient hERG channels with cytosolic-domain mutations tend to preferentially recruit Hsc70 over Hsp70 compared to the WT channel. However, a subset of trafficking-defective mutations in the channel core reduced Hsc70 binding, suggesting a more complicated interplay between specific hERG conformational defects and chaperone/co-chaperone recognition (Li *et al.* 2011).

Hsp90 was initially shown to interact with immature hERG at the ER and to be required for the maturation of hERG and I_{Kr} (Fig. 3) (Ficker *et al.* 2003). More recently, hERG was shown to be preferentially bound by Hsp90 α over Hsp90 β to promote trafficking, highlighting a more

precise relationship for Hsp90 in the folding of hERG (Peterson *et al.* 2012). The specificity of chaperone roles in hERG folding is further complicated by the many co-chaperones that regulate or modify chaperone activity.

hERG co-chaperones. Co-chaperones from the Hsp40 family (DNAJs) and NEFs contribute to the Hsp70/Hsc70 reaction cycle and can regulate chaperone function. DNAJs promote the hydrolysis of ATP into ADP, changing the Hsp70/Hsc70 from an open, substrate-accepting state to a closed, substrate-bound state, and NEFs promote the reverse process by stimulating the exchange of ADP with ATP (Hartl *et al.* 2011).

The impact of DNAJs in substrate folding/degradation seems to be specific to the type of DNAJ and misfolded substrate. It is apparent that while DNAJA1 (Hdj2) and DNAJA2 are highly homologous and are the most abundant DNAJs, they are functionally distinct (Tzankov *et al.* 2008; Baaklini *et al.* 2012). Previous work has shown that DNAJA1 and Hsc70 were both required for CHIP E3 ubiquitin ligase binding to promote degradation of CFTR (Younger *et al.* 2004). Similarly, both DNAJs are thought to promote degradation of hERG through the binding of the pro-degradative Hsc70 chaperone (Walker *et al.* 2010). However, there appear to be functional differences between individual DNAJs. Specifically, knockdown of DNAJA1 and to a lesser extent DNAJA2 also impaired hERG trafficking, suggesting that normal levels of DNAJA1 in particular are needed for Hsp70-assisted folding. (Fig. 3) (Walker *et al.* 2010).

The NEFs consist of three structural families: BAG (Bcl2-associated athanogene 1 related) proteins, the Hsp110 family and HspBP1 (Hsp70 binding protein 1). The NEFs induce the release of the protein substrate from the Hsp70 complex, and under appropriate conditions, can enhance Hsp70-mediated folding (Tzankov *et al.* 2008; Rauch & Gestwicki, 2014). Hsp110s are distinct from the other NEFs because they are thought to bind substrate

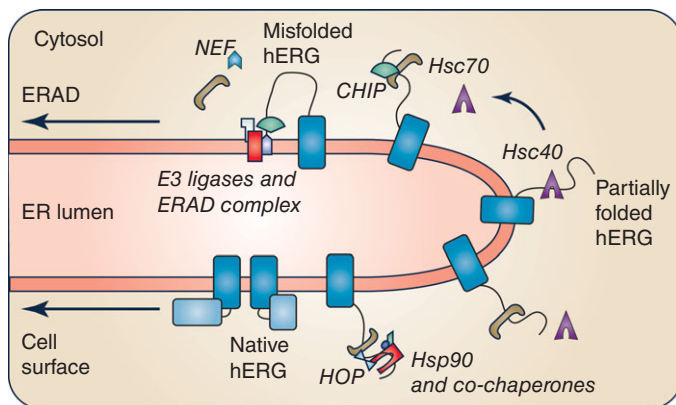


Figure 3. Quality control of misfolded hERG at the ER Partially folded hERG is recognized by Hsp40s such as DNAJA1 and DNAJA2. This binding stimulates the hydrolysis of ATP to ADP, initiating Hsp70/Hsc70 substrate binding and Hsp40 dissociation. DNAJA1 assists folding by subsequent binding of Hsp70, Hsp90 and co-chaperones, resulting in a native form of hERG, which is trafficked to the cell surface. DNAJA2 promotes degradation by initiating CHIP E3 ubiquitin ligase binding. NEFs promote the release of ADP and re-binding of ATP and release of Hsc70 from the substrate, possibly regulating hERG folding or degradation. Chaperone-independent E3 ubiquitin ligases and other proteins may be involved in ER associated degradation (ERAD) of hERG.

directly and are distant homologues of Hsp70 (Xu *et al.* 2008; Saxena *et al.* 2012). Although the roles of NEFs in the folding of other membrane spanning channels have been studied (Young, 2014), their function on hERG is much less explored. One NEF, Bag2, is involved in the Hsp70 cycle of CFTR folding and was also found to associate with hERG (Arndt *et al.* 2005; Walker *et al.* 2007). The exact role of Bag2 on hERG ERQC is not established.

Additional interactions with the substrate protein occur outside the Hsp70 folding cycle, which further complicates the identification of key regulators in hERG protein quality control. One group of co-chaperones are able to bind to Hsc/Hsp70 through tetratricopeptide-repeat (TPR) adaptor domains (Carrigan *et al.* 2006). These proteins can attach various activities to Hsc/Hsp70 or Hsp90 (Young *et al.* 2003, 2004). For example, CHIP is a soluble E3 ubiquitin ligase that promotes degradation of hERG and other substrates bound to Hsc/Hsp70 or Hsp90 in the cytosol and in ERAD (Connell *et al.* 2001; Meacham *et al.* 2001). Another well-characterized TPR co-chaperone is HSP-organizing protein (HOP), which connects Hsc/Hsp70 with Hsp90 so that Hsp90-dependent folding can proceed (Scheufler *et al.* 2000; Pearl & Prodromou, 2006; Li & Buchner, 2013). HOP was found in complex with hERG and may aid the release of hERG from Hsc70 and the recruitment of Hsp90 (Walker *et al.* 2007). Since CHIP and HOP both bind to the same C-terminal motif of Hsc70, the competition between these co-chaperones will result in either degradation or Hsp90 substrate transfer, respectively (Fig. 3) (Scheufler *et al.* 2000; Meacham *et al.* 2001).

It seems logical to use the model of the much more characterized folding cycle of CFTR as an example to target potential co-chaperones involved in hERG folding (Wang *et al.* 2006; Okiyoneda *et al.* 2010), though it is noteworthy to point out that we do not see comparable co-chaperone interactions with hERG to what has been so far described with CFTR (unpublished results). Through enhancing pro-folding co-chaperones and manipulating pro-degradative co-chaperones, it is reasonable to speculate that combined manipulation of Hsc/Hsp70 and co-chaperones could promote the proper folding and trafficking of hERG.

Endoplasmic reticulum associated proteasomal degradation. The chaperone system provides the means to identify misfolded proteins directed for degradation (Hartl *et al.* 2011). However in ERAD, misfolded substrate is polyubiquitinated by an E3 ubiquitin ligase, sometimes independent of chaperones, causing dislocation from the ER followed by unfolding of the substrate and degradation by the 26S proteasome (Hampton & Sommer, 2012). Identification of E3 ligases involved in protein substrate degradation has, therefore, become an interesting area of exploration. In fact, one such cytosolic E3 ligase, CHIP, has

been identified in degrading misfolded proteins including those found at the ER such as CFTR and hERG (Meacham, 2001; Murata *et al.* 2001). The presence of certain members in the Hsp70 folding cycle seems to influence CHIP binding, as seen with some of the DNAs increasing substrate interaction with CHIP, while Hsp90 prevents it, thus indicating that CHIP predominantly acts through Hsp70 (Walker *et al.* 2010; Iwai *et al.* 2013). While CHIP plays a prominent role in hERG degradation, it is recognized that several other ER-resident E3 ligases and ERAD proteins may be involved in hERG degradation (Fig. 3). Indeed, for CFTR, the transmembrane E3 ligases gp78 and RMA1 have been shown to promote degradation of Δ F508-CFTR (Sun *et al.* 2006; Younger *et al.* 2006; Morito *et al.* 2008). A number of proteins including gp78, its homologue E3 ligase HRD1, the derlin family and associated factors are involved in quality control ERAD of other transmembrane proteins as well (Smith *et al.* 2011; Hampton & Sommer, 2012). It seems likely that these E3 ligases and ERAD proteins may also be involved in hERG degradation. The exploration of such ERAD components for hERG have yet to be reported, though they may prove to be an additional mechanism for regulating hERG degradation that could be manipulated for clinical use.

Peripheral quality control

For many years, it was believed that disposal of partially misfolded hERG by the ERQC represents the primary, if not the sole, mechanism for LQT2 pathogenesis (Kuryshv *et al.* 2005; Anderson *et al.* 2006; Rajamani *et al.* 2006; Takemasa *et al.* 2008). However, there is growing evidence that misfolded channels may be trafficked to the cell membrane and their regulation would require proteostasis mechanisms acting in peripheral compartments (Anderson *et al.* 2006; Apaja *et al.* 2013). Consistent with this notion, we recently reported that misfolded hERG channels at the PM are polyubiquitinated and rapidly internalized into the endocytic pathway and targeted for lysosomal delivery (Fig. 4). A similar paradigm was previously described for other misfolded membrane proteins including disease-associated mutant CFTR and G-protein-coupled receptors (Okiyoneda *et al.* 2010; Apaja *et al.* 2013).

Molecular mechanisms responsible for ubiquitination of misfolded hERG at the PM. For several years, the CHIP E3 ubiquitin ligase has been known to ubiquitinate misfolded cytosolic and PM proteins (Okiyoneda *et al.* 2010; Apaja *et al.* 2013). We recently demonstrated that CHIP also promotes the removal of misfolded hERG from the PM via addition of K48- and K63-linked polyubiquitin chains (Apaja *et al.* 2013). Disruption of CHIP binding to Hsc/p70 and Hsp90 prevented the removal of misfolded channels from the PM, suggesting a role of cytosolic chaperones in

recognition of misfolded hERG. In contrast to hERG QC at the ER, the role of cytosolic chaperones/co-chaperones in hERG refolding and/or degradation at the PM remains to be explored. It has been reported that ablation of Hsc70, Hsp90, or co-chaperones Bag1, DNAJB2 (HSJ1), DNAJA1 (Hdj2) and DNAJC7 (Tpr2) prevented removal of misfolded CFTR from the PM (Okiyoneda *et al.* 2010). Interestingly, Hsp70 upregulation did not affect turnover of misfolded CFTR, yet is involved in degradation of other misfolded PM-protein substrates (Apaja *et al.* 2010; Okiyoneda *et al.* 2010). Furthermore, Hsp90 is involved in the degradation of misfolded CFTR, but it conversely stabilizes a large number of conformationally unstable protein kinases at the PM (Taipale *et al.* 2010; Taipale *et al.* 2012). While there are several reports of the roles of chaperone/co-chaperones in other protein folding and stability at the PM, the key modulators for hERG folding and stability at the PM, the key modulators for hERG folding have yet to be established. Interestingly, Hsp90 inhibition did not inhibit the removal of misfolded hERG from the PM (unpublished data). These observations suggest that the role of individual chaperones/co-chaperones in peripheral protein QC can be substrate specific.

Interestingly, CHIP knockdown only partially abolished polyubiquitination and turnover of misfolded PM hERG,

suggesting the involvement of other E3 ligases. Previous studies have implicated Nedd4-2-mediated mono-ubiquitination in the regulation of hERG PM expression under steady-state conditions and in response to kinase serine/threonine-protein kinase (SGK) signalling (Lamothe & Zhang, 2013). However, Nedd4 and Nedd4-2 ablation had no effect on the turnover of misfolded hERG, suggesting that Nedd4-2 is primarily involved in regulating PM hERG in response to second-messenger signalling rather than quality control (Apaja *et al.* 2013; Lamothe & Zhang, 2013). This is supported by the observation that Nedd4-2-mediated ubiquitination is dependent on binding to a C-terminal PY motif on the channel, whereas an E3 ligase involved in quality control would be expected to recruit via hydrophobic regions or cytosolic chaperones (Guo *et al.* 2012).

Internalization and recycling of misfolded hERG. Most PM proteins are targeted for clathrin-mediated endocytosis upon ubiquitination (Piper *et al.* 2014). Interestingly, there is evidence suggesting that hERG is internalized via a clathrin-independent pathway; however, some controversy exists over the exact mechanism. Caveolin was found to be involved in the degradation

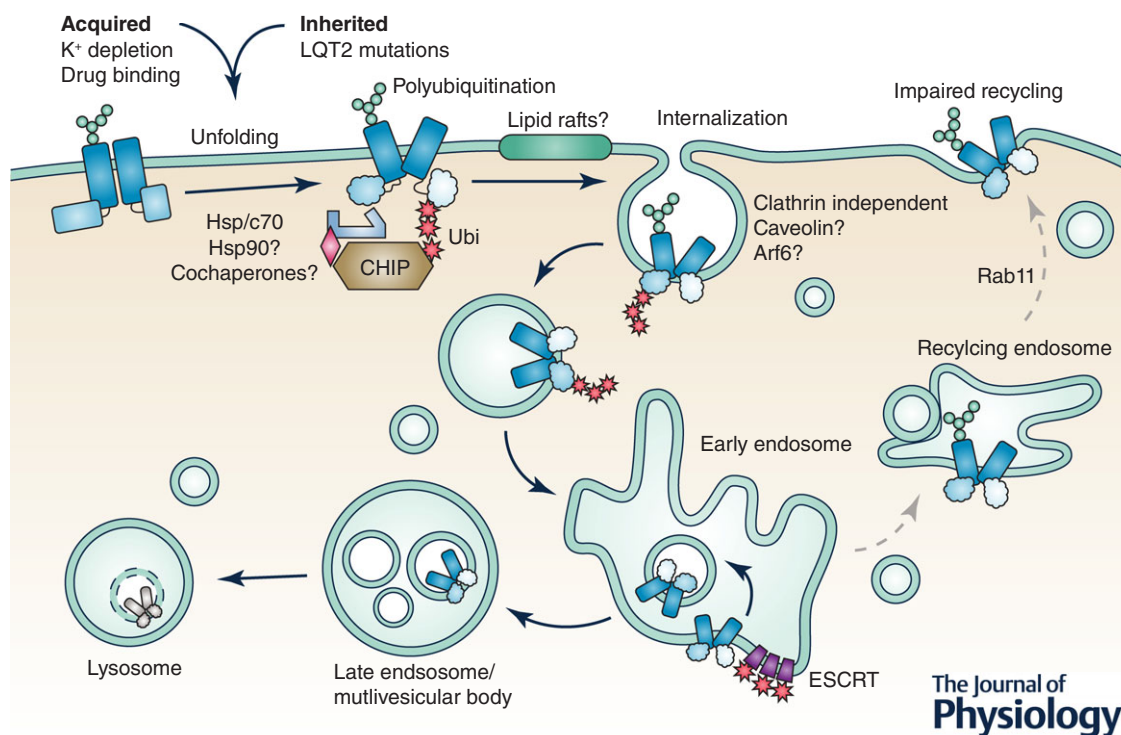


Figure 4. Quality control of misfolded hERG at peripheral cellular compartments

Misfolded hERG cytosolic domains are recognized by cytosolic chaperone proteins such as Hsp/Hsc70 and Hsp90. Chaperone binding results in recruitment of CHIP E3 ubiquitin ligase and subsequent ubiquitination. Polyubiquitin serves as a signal for rapid internalization via a clathrin-independent mechanism. Possible pathways include recruitment to lipid rafts and subsequent caveolin-mediated internalization or an Arf6-dependent mechanism. Internalized proteins may be prevented from recycling back to the plasma membrane and are instead sorted to intraluminal vesicles and targeted for lysosomal degradation by the ESCRT machinery.

of PM hERG in response to unfolding via extracellular K^+ depletion and Nedd4-2-mediated ubiquitination (Guo *et al.* 2012), suggesting that misfolded hERG is internalized via caveolae and lipid rafts. In contrast, a recent study found hERG plasma membrane levels were regulated under steady-state conditions via an Arf6-dependent internalization pathway that is independent of caveolin and dynamin (Karnik *et al.* 2013). Arf6 was shown to be involved in a rapid redistribution of hERG from the PM into endocytic compartments following a short chase (1 h). In contrast, caveolin has only been shown to be involved in degradation of PM hERG over a relatively long time scale (more than 4 h). One interpretation of these data is that the initial internalization step is Arf6 dependent whereas caveolin regulates subsequent endosomal sorting and trafficking processes (Sandvig *et al.* 2011). If this scenario proves to be correct, one could postulate that the internalization of misfolded and ubiquitinated hERG also occurs via an Arf6-dependent mechanism. Another possibility is that internalization is caveolin dependent and Arf6 regulates hERG PM expression through its role in endocytic recycling (Grant & Donaldson, 2009). Further work is required to clarify the hERG internalization machinery, which could represent a novel ubiquitin-dependent clathrin-independent endocytosis pathway. In addition to accelerating internalization, ubiquitination of misfolded hERG was found to impair endocytic recycling. It was recently shown that internalized hERG is recycled by a Rab11-dependent mechanism (Lamothe & Zhang, 2013; Chen *et al.* 2015). It is possible that ubiquitination impairs this process, although the involvement of other recycling pathways for internalized hERG channels cannot be ruled out.

Endosomal sorting for lysosomal delivery. At the endosomal membrane, ubiquitinated cargoes are generally sorted into intraluminal vesicles destined for lysosomal delivery by the endosomal sorting complex for transport (ESCRT) machinery. Lysosomal delivery of misfolded hERG was found to be ESCRT dependent (Sun *et al.* 2011; Apaja *et al.* 2013). Intriguingly monoubiquitination was implicated in the downregulation of PM hERG in response to extracellular K^+ depletion, whereas intracellular K^+ depletion and mutations were found to induce K48- and K68-linked polyubiquitination (Sun *et al.* 2011; Apaja *et al.* 2013). Due to the tetrameric structure of hERG, it is possible that mono/multimono-ubiquitination of several hERG subunits would generate a sufficient sorting signal (Barriere *et al.* 2007). On the other hand, the role of monoubiquitination was established primarily on measurements in cells overexpressing a Lys-free ubiquitin to suppress formation of ubiquitin-linked chains. It is possible that exogenous ubiquitin overexpression artificially promoted substrate mono-ubiquitination. Additionally, even limited incorporation

of endogenous ubiquitin would permit formation of linked chains. In contrast, the observation of hERG poly-ubiquitination was based on direct immunodetection of endogenously ubiquitinated hERG using validated poly-ubiquitin-specific antibodies.

Clinical relevance of hERG QC at the cell periphery. Degradation of hERG by peripheral QC machinery is likely to complement the established ER-exit defect and presents a barrier to the development of therapeutic interventions; however, the relative contribution of these systems remains open to speculation. It is likely that the peripheral QC systems play an important role in acquired LQT2. The plasma concentration of most orally administered drugs peaks shortly after dosage, then declines over time as a result of metabolism and excretion. The turnover of WT-hERG upon biosynthetic inhibition is relatively slow ($T_{1/2} \sim 8\text{--}12$ h (Staudacher *et al.* 2011; Apaja *et al.* 2013). Consequently, it is unlikely that the ERQC machinery alone (which only blocks hERG maturation) will be able to appreciably reduce PM hERG expression before drug plasma concentrations drop to safe levels. On the other hand, removal of misfolded channels from the PM occurs much faster ($T_{1/2} \sim 2$ h), and thus is likely to make a larger contribution to the acquired LQT2 phenotype during the transient period of peak drug concentration (Staudacher *et al.* 2011; Apaja *et al.* 2013). Another intriguing prospect is the possibility that the ER and peripheral QC systems possess a different degree of stringency, with some misfolded hERG conformers recognized by one system but not the other.

Conclusion

The study of hERG in recent years has yielded valuable insights into LQT2 pathogenesis and protein QC pathways in general. The models for protein quality control considered for hERG are based upon recent work on other membrane proteins, in particular CFTR. Although diverse clients may be subject to these same QC pathways, there is a growing appreciation that the underlying molecular machinery may involve a large degree of substrate specificity. In particular, the function of molecular chaperones/co-chaperones to promote either folding or degradation appears to be at least partially dependent on substrate identity and expression levels. It is also likely that additional substrate specificity may exist within the ubiquitination and endocytosis machinery.

For many years, research has been focused on ER QC pathways. Recently, there has been a growing appreciation that the conformation of mature proteins at the PM is policed by an overlapping yet distinct mechanism. This peripheral QC pathway is likely to contribute to the loss-of-expression phenotype in many conformational diseases, including LQT2 and CFTR.

hERG is unique in that misfolding is often induced by pharmacological agents in addition to inherited mutations. Acquired LQT2 is noteworthy in that peripheral QC may represent one of the important mechanisms that contributes to LQT2 due to pharmacokinetic considerations.

Complex interactions may also arise between hERG polymorphisms and approved drugs. This may be an area where personalized medicine may be of use to prevent synergistic interactions between acquired and inherited LQT2 factors. Indeed, adverse interactions have been shown to occur between CFTR, pharmacological correctors and potentiators in a mutation- and patient-specific manner (Cholon *et al.* 2014; Veit *et al.* 2014). Understanding the structural basis of hERG misfolding may provide insights into the interaction between hERG polymorphisms and clinically relevant drugs.

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Additional information

Competing interests

None declared.

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