A forward genetic screen identifies chaperone CNX-1 as a conserved biogenesis regulator of ERG K+ channels

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The human ether-a-go-go–related gene (hERG) encodes a voltage-gated potassium channel that controls repolarization of cardiac action potentials. Accumulating evidence suggests that most disease-related hERG mutations reduce the function of the channel by disrupting protein biogenesis of the channel in the endoplasmic reticulum (ER). However, the molecular mechanism underlying the biogenesis of ERG K+ channels is largely unknown. By forward genetic screening, we identified an ER-located chaperone CNX-1, the worm homologue of mammalian chaperone Calnexin, as a critical regulator for the protein biogenesis of UNC-103, the ERG-type K+ channel in Caenorhabditis elegans. Loss-of-function mutations of cnx-1 decreased the protein level and current density of the UNC-103 K+ channel and suppressed the behavioral defects caused by a gain-of-function mutation in unc-103. Moreover, CNX-1 facilitated tetrameric assembly of UNC-103 channel subunits in a liposome-assisted cell-free translation system. Further studies showed that CNX-1 act in parallel to DNJ-1, another ER-located chaperone known to regulate maturation of UNC-103 channels, on controlling the protein biogenesis of UNC-103. Importantly, Calnexin interacted with hERG proteins in the ER in HEK293T cells. Deletion of calnexin reduced the expression and current densities of endogenous hERG K+ channels in SH-SY5Y cells. Collectively, we reveal an evolutionarily conserved chaperone CNX-1/Calnexin controlling the biogenesis of ERG-type K+ channels.

Introduction

The human ether-a-go-go–related gene (hERG) channel is a delayed rectifier voltage-gated K+ channel that controls repolarization of action potentials in cardiomyocytes (Vandenberg et al., 2012). Dysfunction of hERG is associated with multiple human diseases, including long QT syndrome (LQTS; Curran et al., 1995; Sanguinetti et al., 1995; Trudeau et al., 1995), schizophrenia (Huffaker et al., 2009), epilepsy (Johnson et al., 2009), and multiple types of cancers (Pardo and Stühmer, 2014). Although most LQTS-related hERG mutations result in decreased surface expression of the channel (Harkcom and Abbott, 2010; Anderson et al., 2014), the molecular mechanism underlying maturation of hERG K+ channel is not fully understood.

The sole Caenorhabditis elegans ERG-type K+ channel, UNC-103, is homologous to hERG, with 70% amino acid identity in functionally important transmembrane domains and cyclic nucleotide–binding domain (Garcia and Sternberg, 2003; Li et al., 2017). Previous studies have demonstrated that worm homologues of hERG modifiers, KCR1, Hyperkinetic, and some J-proteins also affect the function of UNC-103 channels (Petersen et al., 2004; Li et al., 2017), suggesting the conserved regulatory mechanisms of ERG K+ channels between C. elegans and humans. Given the convenience of genetic manipulation, C. elegans thus can be used as an animal model to identify novel cellular factors important for regulating ERG channel biogenesis.

In the previous work, through a genetic screen, we found DNJ-1, an ER-located chaperone, is critical for the biogenesis of the UNC-103 K+ channel in C. elegans (Li et al., 2017). Although the expression level of UNC-103 was markedly decreased because of a loss-of-function (LOF) mutation in dnj-1, there were still remaining UNC-103 proteins in the dnj-1 mutant worms, suggesting that other cell factors act independently of DNJ-1 to facilitate the biogenesis of ERG channels. In this study, by forward genetic screening, we identified CNX-1, the C. elegans homologue of mammalian chaperone Calnexin, as a critical factor for the biogenesis of ERG K+ channels.

Calnexin, also known as CNX, IP90, or P88, is an ER-located chaperone with carbohydrate-binding activity (Ware et al., 1995; Schrag et al., 2001). A previous study has demonstrated that a trafficking defect in a LQTS-related mutant, hERG*470D,
is caused by a prolonged association with Calnexin (Gong et al., 2006). This result suggests that Calnexin may be involved in the biogenesis of hERG channels. However, the effect of Calnexin (or its worm homologue, CNX-1) on the biogenesis of ERG K⁺ channels in vivo remains unclear. In this study, we demonstrated that Calnexin/CNX-1 interacted with the ERG channel and facilitated ERG channel biogenesis in the ER. The effect of CNX-1 on the biogenesis of the ERG channel involved its ability to promote tetrameric assembly of the channel subunits. Further experiments showed that the action of CNX-1 was independent of DNJ-1, suggesting that these two different types of chaperones facilitate the maturation of ERG channels via parallel pathways. Thus, we reveal a conserved role of CNX-1 in promoting ERG channel biogenesis.

Materials and methods

Worm strains and culture
OP50 bacteria-seeded nematode growth medium was used to cultivate worms at 20°C. N2, unc-103(e1597), cnx-1(nr2010), and CB4856 strains were obtained from the Caenorhabditis Genetics Center. The dnj-1(yfh0001) mutant was generated in our laboratory previously (Li et al., 2017). For detailed information of all the strains used in this study, please refer to Table 1.

Genetic screens and whole-genome sequencing
Synchronized L3-L4 stage unc-103(e1597) worms were used for mutagenesis. After treatment with 50 mM ethyl methanesulfonate for 4 h at room temperature, the F2 progenies of revertants (mutated worms that can move better and lay eggs) were selected. Hawaii CB4856 was crossed with the revertants to perform genetic mapping of the mutated genes (Davis et al., 2005). Genomic DNA of mutant worms was then prepared for whole-genome sequencing (Doitsidou et al., 2010). Genome sequencing was performed on an Illumina platform (HiSeq 2000/2500 or Hiseq X Ten), and all data were analyzed by CloudMap online software (https://usegalaxy.org). Rescue experiments of LOF alleles of cnx-1 were performed by reexpressing wild-type CNX-1 proteins in cnx-1 mutants.

Behavioral analyses of worms
Thrashing assays were performed as previously described (Petersen et al., 2004). In brief, young adult hermaphrodites were picked in M9 buffer and allowed to recover for 20 s. Thrashes were then counted for 30 s. A movement that the worm swings its head to the same side was counted as a thrash. For egg-laying, late L4 hermaphrodites were individually picked onto an OP50 seeded plate. After 30 h, the total number of the eggs and larvae on the plates was counted. The behavioral studies were not

CGC, Caenorhabditis Genetics Center.

Table 1. Summary of all worm strains used in this study

| Genotype          | Strain     | Description                                                                 |
|-------------------|------------|-----------------------------------------------------------------------------|
| unc-103(e1597)    | CB1597     | GOF worm mutant of unc-103 (from CGC)                                       |
| C. elegans wild isolate | N2        | Bristol C. elegans (from CGC)                                               |
| C. elegans wild isolate | CB4856    | Hawaii C. elegans (from CGC)                                                |
| cnx-1(yfh0017)    | SQC0071    | LOF worm mutant of cnx-1                                                    |
| cnx-1(yfh0018)    | SQC0108    | LOF worm mutant of cnx-1                                                    |
| cnx-1(yfh0019)    | SQC0080    | LOF worm mutant of cnx-1                                                    |
| cnx-1(yfh0020)    | SQC0201    | LOF worm mutant of cnx-1                                                    |
| unc-103(e1597);cnx-1(nr2010) | SQC0115 | unc-103(e1597) crossed with cnx-1(nr2010)                                   |
| unc-103A334T      | SQC0132    | yfhEx0132 (Punc-103::unc103A334T::GFP at 20 ng/µl)                          |
| unc-103A334T; cnx-1(nr2010) | SQC0118 | unc-103A334T crossed with cnx-1(nr2010)                                     |
| unc-103A334T; dnj-1(yfh0001) | SQC0119 | unc-103A334T; dnj-1(yfh0001) crossed with cnx-1(nr2010)                    |
| cnx-1(nr2010)     | NS2932     | LOF worm mutant of cnx-1 (from CGC)                                         |
| dnj-1(yfh0001)    | SQC0001    | LOF worm mutant of dnj-1                                                    |
| cnx-1(nr2010);dnj-1(yfh0001) | SQC0020 | cnx-1(nr2010) crossed with dnj-1(yfh0001)                                  |
| Psp-12::sp12::mCherry; Pcnx-1::cnx-1::GFP | SQC2602 | yfhEx2602 (Pcnx-1::cnx-1::GFP at 50 ng/µl, Psp-12::sp12::mCherry at 50 ng/µl) |
| Pcnx-1::mCherry;Pver-3::GFP | SQC2601 | yfhEx2601 (Pver-3::GFP at 50 ng/µl, Pcnx-1::mCherry at 50 ng/µl)               |
| Punc-103::mCherry;Pcnx-1::GFP | SQC2600 | yfhEx2600 (Pcnx-1::GFP at 50 ng/µl, Punc-103::mCherry at 50 ng/µl)          |
| Pver-3::GFP[N2]   | SQC0124    | ALA neuron labeling in N2 (Pver-3::GFP at 30 ng/µl)                          |
| Pver-3::GFP[cnx-1(nr2010)] | SQC0125  | ALA neuron labeling in cnx-1(nr2010) (Pver-3::GFP at 30 ng/µl)               |
| Pver-3::GFP[dnj-1(yfh0001)] | SQC0126  | ALA neuron labeling in dnj-1(yfh0001) (Pver-3::GFP at 30 ng/µl)             |
| Pver-3::GFP[cnx-1(nr2010); dnj-1(yfh0001)] | SQC0127 | ALA neuron labeling in cnx-1(nr2010); dnj-1(yfh0001) (Pver-3::GFP at 30 ng/µl) |
Figure 1. A forward genetic screen identifies CNX-1 as a biogenesis regulator of UNC-103 K⁺ channel. (A) Sequence alignment of transmembrane and cyclic nucleotide binding domains (cNBD) of mouse ERG, hERG, C. elegans UNC-103, and Drosophila Seizure channels. Identical amino acids are highlighted in yellow. Transmembrane and cyclic nucleotide binding domains are underlined. The triangle indicates the position of the GOF mutation in unc-103. (B) Schematic strategy of forward genetic screening. Mutagenesis was performed by treating unc-103(e1597) worms with ethyl methanesulfonate (EMS). EMS-induced mutants with recovery from behavioral defects were selected. SNP, single-nucleotide polymorphism; WGS, whole-genome sequencing. (C) Cloning of cnx-1. Chr III, chromosome III. Red lines indicate the mutated sites in exons and introns of the cnx-1 gene. The deleted DNA fragment of cnx-1 in cnx-1(nr2010) worms is indicated. (D) Sequence alignment of CNX-1 and its human homologue, Calnexin. Identical amino acids are shown in yellow. Arrows point to the mutated alleles in CNX-1.
conducted blind to genotypes, but two researchers independently did the experiments and obtained similar results.

### Molecular biology and generation of transgenic worms

P<sub>cnx-1::gfp</sub> and P<sub>unc-103::mCherry</sub> were generated by inserting 2.2 kb cnx-1 promoter and 4.3 kb unc-103 promoter into the pPD95.75 vector, respectively, and then coinjected into N2 worms to analyze the expression pattern of cnx-1. ER marker sp12 cDNA and cnx-1 DNA were individually fused with the cnx-1 promoter and then inserted into the pPD95.75 vector to generate P<sub>cnx-1::cnx-1::gfp</sub> and P<sub>cnx-1::sp12::mcherry</sub> plasmids. These two plasmids were coinjected into N2 worms to analyze the subcellular localization of CNX-1. P<sub>ver-3::gfp</sub> was injected into N2, cnx-1(nr2010), dnj-1(yfh0001), or cnx-1(nr2010);dnj-1(yfh0001) worms to label the ALA neurons in these worm strains. For detailed information about these transgenic worms, please see Table 1.

### Western blot and immunoprecipitation

HEK293T and SH-SYSY cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco) with 10% FBS (Gibco), and 1% penicillin/streptomycin (Gibco) at 37°C in a 5% CO<sub>2</sub> incubator. Lipofectamine 2000 (Invitrogen) was used for plasmid transfection. The components of cell lysis buffer were 1% NP-40, 150 mM NaCl, 1 mM NaF, 50 mM Tris, pH 7.6, and protease inhibitor mixture (Roche). Cells were lysed in lysis buffer at 4°C for 1 h. After centrifugation at 4°C at 12,000 g for 10 min, the supernatants were collected and incubated with the primary antibody at 4°C for 3.5 h. The immune complexes were associated with protein A/G agarose beads (Santa Cruz) at 4°C for 2.5 h. The beads were then washed six times and boiled for 5 min. The Western blot analyses of UNC-103 protein levels were not conducted blind to genotypes, but two researchers independently did the experiments and obtained similar results. The following antibodies were used: rabbit anti-hERG (Millipore), rabbit anti-c-Myc (Sigma), rat anti-HA (Roche), mouse anti-tubulin (Sigma), mouse anti-GFP (Abmart), and rabbit anti-actin (Abmart).

### RNA interference

For knockdown experiments by siRNA, siRNAs were first transfected into HEK293T cells and then cotransfected with HA-hERG plasmid 16 h later. The scrambled (control) siRNA and human calnexin siRNA were purchased from Genepharma. The sequence of the control siRNA was 5'-UUCUCCGAACGUCGACUUTT-3' and 5'-AACACAGUGACACGUUGUCUTT-3'. Two siRNAs corresponding to the human calnexin coding sequences (5'-AAGAGTACCGATGATAAAG-3' and 5'-AATGGTGGTGGTGCTATGTA-3') were used according to the previous study (Swanton et al., 2003).

### Table 2. Detailed information for different cnx-1 alleles

| Gene | Strain | Allele | Effect | Gene change | Position |
|------|--------|--------|--------|-------------|----------|
| cnx-1 | SQC0071 | yfh0017 | Stop codon | tG6/tA6 | Exon 1 |
| cnx-1 | SQC0108 | yfh0018 | Stop codon | tG6/tA6 | Exon 3 |
| cnx-1 | SQC0080 | yfh0019 | Insertion | Insertion t | Exon 4 |
| cnx-1 | SQC0201 | yfh0020 | Splicing site | G1915A | Last base pair in intron 5 |
Clustered regulatory interspace short palindromic repeats (CRISPR)-Cas9–mediated genome editing

SH-SY5Y cell line with deletion of calnexin was generated by CRISPR-Cas9–mediated genome editing according to the standard protocol described previously (Ran et al., 2013). We used four sgRNAs targeting four loci of calnexin to induce DNA double strand breaks. sgRNAs were individually cloned into the CRISPR vector. The sequences of sgRNAs were 5'-GCTTGGAGCTGCTATTGTG-3', 5'-CCCTTCATAAGACATCGT-3', 5'-TTTGACAGAGGAAGCTCTGTC-3', and 5'-TATACCTCCCTGGTGAAC-3'. Calnexin+/− cells were verified by DNA sequencing (the PCR primers were forward, 5'-GTTTAAACGGGTATCCCCCTGTG-3' and reverse, 5'-AGAGTGGGGGAAGA-3'), followed by Western blot analysis using Calnexin antibody (Abcam) to detect Calnexin proteins in the cells.
Electrophysiology

The K⁺ currents of ERG channels in cells were recorded by whole-cell patch clamp as described previously (Li et al., 2017). Pipette resistances were 5 MΩ for recording in HEK293T cells and SH-SY5Y cells. Pipette resistances were 15 MΩ for recording in C. elegans ALA neurons. The pipette solution for patch clamp in HEK293T cells contained (in mM) 100 KCl, 1 MgCl₂, 1 CaCl₂, 10 EGTA, and 10 HEPES, pH 7.5 (with KOH); and bath solution for HEK293T cells contained (in mM) 4 KCl, 100 NaCl, 10 HEPES, 1 MgCl₂, and 1.8 CaCl₂, pH 7.5 (with NaOH). For SH-SY5Y cells, the pipette solution was (in mM) KCl 135, MgCl₂ 1, and EGTA 10, HEPES 10, pH 7.2 (with KOH); and bath solution was (in mM)
KCl 5, NaCl 130, glucose 10, MgCl₂ 1, and CaCl₂ 2, HEPES 10, pH 7.4 (with NaOH). For C. elegans ALA neurons, the bath solution contained (in mM) 5 KCl, 145 NaCl, 1 CaCl₂, 5 MgCl₂, 10 HEPES/NaOH, pH 7.5, and 20 d-glucose. The pipette solution contained (in mM) 18 KCl, 125 potassium gluconate, 0.7 CaCl₂, 2 MgCl₂, 10 EGTA/KOH, 2 Mg-ATP, and 10 HEPES/KOH, pH 7.5. ERG K⁺ currents were isolated by application of 1 µM E-4031 in the pipette solution.

Statistical analyses
The band intensities in immunoblots were determined by ImageJ software. The amplitudes of K⁺ currents and the membrane capacitances of the recorded cells were measured using Clampfit software (version 10.4; Molecular Devices). Peak currents and steady-state currents at each voltage step were used for statistical analyses. Graphing and statistical comparisons were performed with GraphPad 7.0 software using either ANOVA or t test as specified in the figure legends.

Results
LOF mutations of cnx-1 suppress the behavioral defects in unc-103(GOF) worms
The C. elegans ERG K⁺ channel UNC-103 showed homology with the hERG channel (Fig. 1A). unc-103(e1597) mutant worms exhibit severe defects in locomotion and egg-laying caused by a gain-of-function (GOF) mutation (A334T) in the S6 transmembrane domain of UNC-103 (Garcia and Sternberg, 2003; Reiner et al., 2006). The mutation is a GOF allele that alters the channel activation potential to a more negative value (Petersen et al., 2004) and inhibits the excitability of the cell (Collins and Koelle, 2013). We thus hypothesized that inhibition of the function of UNC-103 will alleviate the behavioral defects in unc-103(e1597) worms. To identify novel regulators of ERG K⁺ channels, we performed a forward genetic screen for suppressors of the behavioral defects of unc-103(e1597) worms. The screening strategy is depicted in Fig. 1B.

From a screen of 14,000 haploid genomes, we isolated eight mutants with reverted phenotypes. Four of the isolated mutants were mapped to one gene, cnx-1 (ZK632.6), which encodes a chaperone protein showing homology with mammalian Calnexin, through single-nucleotide polymorphism–based mapping (Fig. 1, C and D). Molecular lesions were identified in all the four cnx-1 alleles through DNA sequencing (Fig. 1D and Table 2). As shown in Fig. 2 (A–C), the behavioral defects in locomotion (quantified by thrashing) and egg laying (quantified by egg numbers) found in unc-103(e1597) worms were partially prevented by a LOF mutation or a deletion allele (nr2010) of the cnx-1 gene. We then performed rescue experiments by reexpressing the wild-type CNX-1 with the driver of its own promoter in unc-103(e1597);cnx-1(nr2010) worms and found that reintroduction of the wild-type CNX-1 could reinstate the behavioral defects of unc-103(e1597) (Fig. 2, B and C), demonstrating that the lack of the CNX-1 protein, but not
other mutations in the genetic background, is responsible for the observed phenotypes. Together, these results suggest that CNX-1 is essential for the function of the UNC-103 K+ channel in *C. elegans.*

**CNX-1 is required for UNC-103 biogenesis**

Our previous study generated a transgenic strain (named unc-103A334T) expressing the GFP fused unc-103 with the same GOF mutation found in unc-103(e1597) worms (Li et al., 2017). Like unc-103(e1597), the transgenic worm exhibited severe defects in locomotion and egg laying, indicating that expression of UNC-103A334T::GFP was sufficient to induce functional defects caused by the UNC-103 mutation, presumably by causing membrane hyperpolarization after its delivery to the plasma membrane.

To test whether CNX-1 regulates UNC-103 protein biogenesis, we crossed unc-103A334T transgenic worms with the *cnx-1(nr2010)* worms to obtain the unc-103A334T,cnx-1(nr2010) strain. We found that the expression level of UNC-103A334T::GFP was significantly reduced in unc-103A334T,cnx-1(nr2010) worms compared with unc-103A334T worms (Fig. 3, A and B). Furthermore, the behavioral defects of unc-103A334T transgenic worms were significantly suppressed by the LOF mutation in *cnx-1* (Fig. 3, C and D, left). Thus, these results suggest that CNX-1 is required for the protein biogenesis of UNC-103 in *C. elegans.*

Formation of functional K+ channels requires channel subunits assembly, an important biogenic step occurring in the ER (Doyle et al., 1998; Isacoff et al., 2013). We then explored whether CNX-1 plays a role in the tetrameric assembly of UNC-103 channel subunits using a wheat germ cell-free system to synthesize proteins in vitro (Fig. 4 A). UNC-103 and CNX-1 were expressed in the cell-free extracts with liposomes that provide a hydrophobic...
Figure 6. CNX-1 regulates UNC-103 K⁺ currents. (A) Fluorescent images of transgenic worms expressing Pver-3::GFP and Pcnx-1::mCherry. Arrows indicate ALA neurons. Bar, 10 µm. (B) Schematic illustration of primary culture of C. elegans neurons for electrophysiological recordings. Whole-cell patch clamp was performed 4–6 d after seeding C. elegans embryonic cells. (C) Representative whole-cell K⁺ currents recorded in C. elegans ALA neurons with or without the
environment to help the folding and assembly of these membrane proteins (Goren et al., 2009). We found that in vitro–synthesized UNC-103 existed as monomers, whereas part of the UNC-103 proteins appeared as tetramers upon coexpression with CNX-1 (Fig. 4, B and C), indicating that CNX-1 promotes tetrameric assembly of UNC-103 channel subunits in vitro.

The effects of CNX-1 and DNJ-1 on UNC-103 protein biogenesis are additive

Previous studies have demonstrated that DNJ-1, another ER-located chaperone protein, is critical for the biogenesis of the UNC-103 K⁺ channel (Li et al., 2017). We then asked whether CNX-1 and DNJ-1 regulate the biogenesis of UNC-103 via the same pathway. We found that the expression level of UNC-103A334T::GFP was markedly reduced by introducing either null mutations in cnx-1 or dnj-1 and almost abolished by introducing both the cnx-1 and dnj-1 mutations (Fig. 3, A and B). The locomotion and egg-laying defects found in unc-103A334T worms could be partially prevented by null mutations in either cnx-1 or dnj-1 and further prevented by a combination of cnx-1 and dnj-1 null mutations (Fig. 3, C and D, left). Notably, locomotion and egg production in cnx-1(nr2010);dnj-1(yfh0001) worms were not significantly different from those in unc-103A334T;cnx-1(nr2010);dnj-1(yfh0001) worms, suggesting that the combination of cnx-1 and dnj-1 null mutations fully suppressed the locomotion and egg-laying defects caused by the GOF mutation in UNC-103. Thus, the effects of CNX-1 and DNJ-1 on UNC-103 channel biogenesis are additive.

CNX-1 regulates the function of endogenous UNC-103 K⁺ channels

To determine the subcellular localization of CNX-1 proteins, we fused GFP and mCherry to the C termini of CNX-1 and the ER marker protein SP12, respectively. We found that CNX-1 was colocalized with SP12 in C. elegans neurons (Fig. 5 A), indicating that CNX-1 is an ER-located protein. Further study showed that cnx-1 promoter–driven GFP was ubiquitously expressed in large numbers of neurons in the tail, head, and vulva (Fig. 5 B) and colocalized with unc-103 promoter–driven mCherry in many neurons (Fig. 5 B), suggesting that CNX-1 and UNC-103 may interact with each other in vivo.

Furthermore, cnx-1 promoter–driven mCherry proteins were colocalized with the ALA-specific ver-3 promoter–driven GFP, suggesting that CNX-1 was expressed in ALA neurons (Fig. 6 A). To investigate whether CNX-1 regulates endogenous UNC-103 K⁺ channels, we examined the effect of CNX-1 on UNC-103 function in ALA neurons, where UNC-103 is expressed (Li et al., 2017). We recorded outward K⁺ currents in cultured C. elegans ALA neurons, which were labeled by ALA-specific ver-3 promoter–driven GFP (Fig. 6 B). Endogenous UNC-103 currents were isolated by E-4031, an ER K⁺ channel–specific blocker (Fig. 6 C). Compared with those in wild-type N2 worms, E-4031–sensitive currents of ALA neurons in cnx-1(nr2010) worms were reduced (Fig. 6, D and E), indicating that CNX-1 regulates endogenous UNC-103 K⁺ channels in C. elegans. Furthermore, we found that both dnj-1 and cnx-1 mutant worms showed decreased UNC-103 currents (isolated by E-4031) in ALA neurons (Fig. 6, D and E), whereas the UNC-103 currents in cnx-1(nr2010);dnj-1(yfh0001) double-mutant worms were eliminated (Fig. 6, D and E). These results further suggest that CNX-1 and DNJ-1 regulate the function of the UNC-103 K⁺ channel independent of each other.

Calnexin regulates the biogenesis of hERG K⁺ channel in mammalian cells

C. elegans CNX-1 is homologous with mammalian protein Calnexin. We thus investigated whether Calnexin plays an evolutionarily conserved role in regulating the biogenesis of hERG channels. Immunostaining in HEK293T cells showed that hERG proteins distributed in both the cell surface and the ER (Fig. 7 A). Consistent with the previous studies (Zhou et al., 1998; Anderson et al., 2006), we found two forms of hERG proteins corresponding to ER (~135 kD) and post-ER (~155 kD) forms with different extents of glycosylation (Fig. 7 B). Coimmunoprecipitation assays showed that Calnexin only interacted with the ER-residing form of hERG proteins (Fig. 7 B), suggesting that Calnexin interacts with hERG proteins in the ER.

We next investigated whether hERG K⁺ channels could be regulated by Calnexin. In HEK293T cells expressing hERG proteins, down-regulation of calnexin by siRNA markedly decreased the protein level (Fig. 7 C) and current density (Fig. 7 D) of hERG K⁺ channels. In human neuroblastoma SH-SY5Y cells that endogenously express hERG K⁺ channels, deletion of calnexin by CRISPR-Cas9–mediated genome editing significantly decreased the endogenous hERG protein level (Fig. 7 E). Furthermore, the deactivating inward currents, which are mainly conducted by hERG channels (Meyer and Heinemann, 1998; Taglialetela et al., 1998; Li et al., 2017), were significantly reduced in calnexin−/− SH-SY5Y cells compared with wild-type SH-SY5Y cells (Fig. 7 F). Collectively, these findings suggest that Calnexin is essential for the protein biogenesis of native hERG channels in human cells.

Discussion

In this study, through forward genetic screen in C. elegans, we found that CNX-1 is a critical regulator of the UNC-103 K⁺ channel. LOF mutations of cnx-1 resulted in a reduced protein level...
and function of UNC-103 K+ channels in C. elegans. Down-regulation of its human homologue, Calnexin, significantly decreased the protein level and current density of the hERG K+ channel in human SH-SYSY cells. Thus, our findings reveal an evolutionally conserved role of CNX-1/Calnexin in regulating ERG-type K+ channels across species.
Calnexin has been shown to play an important role in the protein folding of many secreted proteins, including ion channels (Gong et al., 2006; Li et al., 2010). G protein–coupled receptors (Free et al., 2007; Markkanen and Petäjä-Repo, 2008; Noorwez et al., 2009), MHC class I molecules (Ireland et al., 2007), and the nicotinic acetylcholine receptor (Chang et al., 1997). It has been also suggested that Calnexin facilitates the assembly of channel subunits into a stable complex (Chang et al., 1997; Green, 1999), but so far, there is no direct experimental evidence to support this idea. In this study, our findings show that CNX-1 facilitates biogenesis of ERG K+ channels in C. elegans and promotes tetrameric assembly of ERG K+ channels in a cell-free translation system, providing direct evidence to support this notion. However, the detailed mechanism underlying how Calnexin facilitates the biogenesis of ERG K+ channels remains to be further studied.

Two major subtypes of chaperones exist in the ER: classical HSP family chaperones and carbohydrate-binding chaperones (Braakman and Hebert, 2013). It is generally thought that these two different types of chaperones cooperate during protein biogenesis to ensure adequate protein flux through the ER (Braakman and Hebert, 2013), but how they work together to control protein biogenesis remains largely unknown. Here, we demonstrate that two types of ER-located chaperones, Calnexin and J-proteins, facilitate the biogenesis of ion channels via distinct mechanisms, providing new insight into the role of ER-located chaperones in protein homeostasis.

The biogenesis of ion channels in the ER involves several steps, including glycosylation, folding, assembly, quality control, and ER exit. Besides Calnexin and DNAJB12/DNAJB14, some other chaperone proteins, including HSP70/HSC70 (Li et al., 2011), HSP90 (Ficker et al., 2003), DNAJ1/DNAJ2 (Walker et al., 2010), sigIR (Crottès et al., 2011), and FKBP38 (Walker et al., 2007) have been reported to be involved in the quality control, ER exit, or degradation of hERG. These chaperones may act at different stages of ERG channel biogenesis with distinct molecular mechanisms. This notion was supported by the findings that DNJ-1 and CNX-1 act in parallel to regulate the biogenesis of ERG K+ channels, and previous studies have reported that different trafficking-defective hERG mutants show abnormal binding with different chaperones (Delisle et al., 2004; Curran and Mohler, 2015). Further dissection of the role of chaperones in ion channel biogenesis will facilitate our understanding of the channellopathies caused by defective biogenesis of ion channels.

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