An N-Terminal ER Export Signal Facilitates the Plasma Membrane Targeting of HCN1 Channels in Photoreceptors

Yuan Pan, Joseph G. Laird, David M. Yamaguchi, and Sheila A. Baker

Department of Biochemistry, Carver College of Medicine, University of Iowa, Iowa City, Iowa, United States

Correspondence: Sheila A. Baker, Department of Biochemistry, 4-712 BSB, University of Iowa, 51 Newton Road, Iowa City, IA 52242, USA; sheila-baker@uiowa.edu.

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Purpose. Hyperpolarization-activated cyclic nucleotide-gated 1 (HCN1) channels are widely expressed in the retina. In photoreceptors, the hyperpolarization-activated current (I_h) carried by HCN1 is important for shaping the light response. It has been shown in multiple systems that trafficking HCN1 channels to specific compartments is key to their function. The localization of HCN1 in photoreceptors is concentrated in the plasma membrane of the inner segment (IS). The mechanisms controlling this localization are not understood. We previously identified a di-arginine endoplasmic reticulum (ER) retention motif that negatively regulates the surface targeting of HCN1. In this study, we sought to identify a forward trafficking signal that could counter the function of the ER retention signal.

Methods. We studied trafficking of HCN1 and several mutants by imaging their subcellular localization in transgenic *X. laevis* photoreceptors. Velocity sedimentation was used to assay the assembly state of HCN1 channels.

Results. We found the HCN1 N-terminus can redirect a membrane reporter from outer segments (OS) to the plasma membrane of the IS. The sequence necessary for this behavior was mapped to a 20 amino acid region containing a leucine-based ER export motif. The ER export signal is necessary for forward trafficking but not channel oligomerization. Moreover, this ER export signal alone counteracted the di-arginine ER retention signal.

Conclusions. We identified an ER export signal in HCN1 that functions with the ER retention signal to maintain equilibrium of HCN1 between the endomembrane system and the plasma membrane.

Keywords: hyperpolarization gated channels, photoreceptor, neuron, retina, inner segment, ER export, motif, signal targeting, localization, forward-traffic motifs, plasma membrane, voltage-dependent K^+ channels

Hyperpolarization-activated cyclic nucleotide-gated channels (HCN1, HCN2, HCN3, and HCN4) are differentially expressed in the retina and brain. They are important for multiple neuronal functions including shaping resting membrane potential, modulating synaptic output, and dendritic integration.[1] HCN1, which is most abundant in the inner segment (IS) of rods and cones, carries a feedback current that shapes vision at mesopic and photopic conditions.[2–4]

Trafficking is one of the key regulatory mechanisms for HCN1 function, exemplified by the altered HCN1 trafficking observed in CA1 pyramidal neurons after temporal lobe epilepsy.[5] HCN1 trafficking involves a series of signals that are beginning to be unraveled. The large intracellular C-terminal domain of HCN1 is a hot-spot for trafficking signals. This region has two binding sites for the tetratricopeptide repeat-containing Rab8b interacting protein (TRIP8b), an accessory subunit of HCN channels.[1] TRIP8b maintains the concentration of HCN1 at the distal dendrites in hippocampal CA1 and neocortical layer V neurons.[6–10] However, neither HCN1 compartmentalization nor surface expression in the retina is TRIP8b-dependent.[11] The C-terminus of HCN1 also contains the cyclic nucleotide binding domain (CNBD) common to all HCN channels. The CNBD was shown to regulate the surface expression of HCN2 and may participate in the trafficking of other HCN channels.[12] Following the CNBD, there is a binding site to filamin-A, which is required for clustering HCN1 at the cell surface and promotes HCN1 internalization in hippocampal neurons.[13,14] We have previously identified a di-arginine endoplasmic reticulum (ER) retention signal at the C-terminus of HCN1 using *X. laevis* rod photoreceptors.[15] This ER retention signal negatively regulates the surface expression of HCN1. However, the process of overcoming the retention signal under physiological conditions remains unclear.

An ER retention signal is usually counteracted by a forward trafficking signal that promotes the movement of the protein from the endomembrane system to the surface (i.e., plasma membrane). However, the overall effect of fusing the HCN1 C-terminus on a reporter membrane protein is to retain the reporter in the ER. This suggests that the forward trafficking signal is present in other regions of HCN1. One candidate for mediating the forward trafficking signal is protocadherin 15, which interacts with the N-terminus (NT) of HCN1 in inner ear cells.[16] However, HCN1 and protocadherin 15 do not colocalize in photoreceptors[17,18]; raising the question as to which other HCN1 trafficking signals or regulators may promote its trafficking in this cell.

The goal of this study was to identify novel forward trafficking signals using the established transgenic *X. laevis* approach for probing membrane protein trafficking pathways.[19] We found that in rods, the intracellular NT of HCN1 is necessary for the protein to target the IS plasma membrane

(ISPM). Through investigating a series of truncation mutants, we identified a leucine-based ER export signal that can override the di-arginine ER retention signal. This finding of combinatorial trafficking signals controlling HCN1 localization provides insight into how the amount of HCN1 functioning at the cell surface is regulated under normal and disease conditions.

Methods

Molecular Cloning

The *X. tropicalis* sequence of HCN1 was used to make all constructs expressed in transgenic frogs; inserts were subcloned in the XOP5.5 vector. Mouse HCN1 sequence was used to make the constructs expressed in HEK293 cells; inserts were subcloned in the pEGFPN1 vector. All subcloned inserts of HCN1 were generated by standard PCR protocols and verified by Sanger sequencing (Iowa Institute of Human Genetics, University of Iowa, Iowa City, IA, USA).[15]

Animals

Xenopus laevis were purchased from Nasco (Fort Atkinson, WI, USA) and maintained by the Office of Animal Research at the University of Iowa. All experiments were approved by the Institutional Animal Care and Use Committee and adhered to the ARVO guidelines for animal use in vision research. Transgenic *X. laevis* tadpoles were generated using restriction enzyme mediated integration as previously described.[20,21] Embryos were housed in 0.3× Marc's Modified Ringer (30 mM NaCl, 0.6 mM KCL, 0.3 mM $MgCl_2$, 0.6 mM $CaCl_2$, and 1.5 mM HEPES, pH 7.4). Transgenic tadpoles were identified at St 42 by screening for GFP expression in the eye and humanely euthanized between St 45 and 55 by immersion in 0.2% tricaine (Sigma-Aldrich Corp., St. Louis, MO, USA), prior to processing for immunohistochemistry.

Immunohistochemistry

Immunohistochemical staining of transgenic tadpole retinas was conducted as described previously.[15] Transgenic tadpoles were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA), cryoprotected in 30% sucrose, and frozen in Tissue-Tek O.C.T (Electron Microscopy Sciences, Hatfield, PA, USA). Sections collected on charged glass slides were permeablilized in 0.5% Triton X-100, blocked with 5% goat serum and 0.5% Triton X-100 in PBS, incubated in mouse α-GFP antibodies (diluted 1:5; 4C9 DSHB, University of Iowa) or rabbit α-calnexin antibodies (diluted 1:100; Enzo Life Sciences, Farmingdale, NY, USA), followed by secondary goat α-rabbit or goat α-mouse antibodies conjugated to Alexa 488 or 568 (Life Technologies, Grand Island, NY, USA) mixed with 2 μg/mL Hoechst 33342 (Life Technologies) to label the nuclei. Images were collected using a Zeiss 710 confocal microscope (Central Microscopy Research Facility, University of Iowa). Manipulation of images was limited to adjusting the brightness and contrast levels using Zen Light 2009 (Carl Zeiss Microscopy, Jena, Germany) or Photoshop (Adobe Systems, Inc., San Jose, CA, USA). A minimum of four individual transgenic tadpoles were studied for every DNA construct.

Velocity Sedimentation

HEK293 cells (ATCC, Manassas, VA, USA) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Life Technologies). Cells grown in T75 flasks to confluence were transfected with 9 μg plasmid DNA using Lipofectamine 2000 (Life Technologies). Forty-eight hours post transfection, the cells were lysed in PBS containing 1% CHAPS and 2× protease inhibitor cocktail (Roche, Mannheim, Germany). After clarification by centrifugation at 10, 000*g* for 15 minutes, the lysates were layered on a 5% to 20% continuous sucrose gradient and centrifuged at 209,500*g* for 16 hours at 4°C. 0.5-mL fractions were collected. Proteins (~8%) were fractionated on 10% Mini-PROTEAN TGX gels and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked in 5% milk and incubated with the following primary antibodies: rabbit α-HCN1 (diluted 1:3000)[11] and mouse α-NKA (diluted 1:100; M7-PB-E9; Santa Cruz Biotechnology, Dallas, TX, USA) and secondary antibodies conjugated to HRP. Blots were incubated with SuperSignal West Femto Maximum Sensitivity Substrates (Thermo Fisher Scientific, Waltham, MA, USA) and visualized with a CCD camera (ImageQuant LAS; GE Healthcare, Little Chalfont, Buckinghamshire, UK). The software package Image Studio v3.1 (LI-COR Biosciences, Lincoln, NE, USA) was used for analysis of the images.

Results

The N-terminus is Required for HCN1 to Target the ISPM of *X. laevis* Photoreceptors

In order for HCN1 to reach the plasma membrane, the di-arginine ER retention motif should be balanced by the action of an as yet unknown forward trafficking signal. HCN1 has four intracellular domains all of which are spatially available to trafficking regulators; the NT loop 1 connecting transmembrane domains 2-3, loop 2 connecting transmembrane domains 4-5, and the C-terminus (Fig. 1A). We fused each of these HCN1 domains to a palmitoylated GFP reporter and examined their subcellular localization when expressed in rods of transgenic *X. laevis*. For reference, the design and results from all HCN1 constructs generated in this study are summarized in the Table. The membrane reporter consists of EGFP followed by a palmitoylated peptide consisting of amino acids 311 to 349 from *X. laevis* rhodopsin (Fig. 1B) and has been used in multiple previous studies of protein trafficking in this model system. When expressed alone, the reporter localizes mainly to the outer segment (OS) with some variable but relatively minor levels of signal in the ISPM or ER/Golgi compartments (Fig. 1C).[22,23] Consistent with our previous finding using a similar integral membrane reporter, fusing the C-terminus (CT) of HCN1 to the reporter redirects the protein to the ER (Fig. 1D).[15] Fusion of loop 1 (L1) did not affect the default localization of the reporter (Fig. 1E). Fusion of loop 2 (L2) redirected the reporter to the ellipsoid (Fig. 1F). We did not pursue this observation further because full-length HCN1 is not associated with mitochondria. Fusion of the NT of HCN1 to the reporter prevented it from localizing to OS. It was found in the IS with variable localization to both internal membranes with morphology consistent with the ER and Golgi, and to the ISPM (Fig. 1G; note: our use of the term ISPM encompasses the plasma membrane surrounding all components of the IS [calycal processes, ellipsoid, myoid and soma, axon, and syntaptic terminal]). This indicates that the NT of HCN1 contains trafficking information.

Because the NT only displays partial ISPM targeting when fused to the reporter, we brought back other elements in HCN1 to test if the NT is required for the targeting of full-length HCN1 to the ISPM. When the full-length GFP-tagged *Xenopus tropicalis* HCN1 (aa 1-839) was expressed in transgenic *X. laevis* photoreceptors, the majority of the protein was found at the ISPM (Fig. 2A). HCN1 lacking the NT (GFP-HCN1; aa122-839) was retained in the ER, evidenced

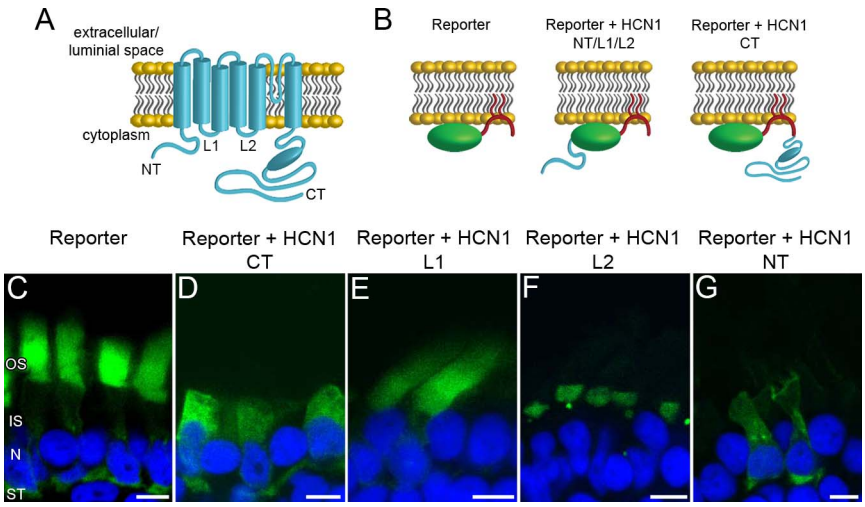


Figure 1. The NT drives a membrane-associated reporter to the ISs. (**A**) Schematic representation of the topology of HCN1. The HCN1 monomer (*cyan*) has six transmembrane domains and four cytoplasmic regions including the NT, the CT, and two intracellular loops (L1 and L2). The *cyan oval* in the CT indicates the relative position of the CNBD. (**B**) Schematic demonstration of the reporter-based constructs studied. The cartoon of the reporter (*left*) consists of a GFP (*green*) and a palmitoylated peptide (*red*). Positions where the cytoplasmic regions of HCN1 (*cyan*) were attached to the reporter are shown in the middle and the right. (**C**–**G**) Confocal images of transgenic *X. laevis* rod photoreceptors expressing the indicated Reporter-HCN1 fragments detailed in Table. N (*blue*), nuclei; ST, synaptic terminals. *Scale bars*: 5 μm.

by colocalization with calnexin, an ER marker (Figs. 2B–D). Several examples of cells expressing wild-type or NT-truncated HCN1 are shown in Supplementary Figure S1, with line scans of the GFP fluorescence intensity across the cell, which demonstrates that the lack of the NT resulted in greater retention within the internal membranes of the cell. In total, these data show that the NT of HCN1 contains an ISPM targeting signal.

Identification of an ER Export Motif in HCN1

We next mapped the NT targeting signal. The localization of a series of N-terminal truncation mutants of GFP-HCN1 were analyzed (Fig. 3; Table). GFP-HCN1 still localized to the ISPM with the first 34, 54, or 74 amino acids (aa) deleted (Figs. 3A–C). Removal of the first 94 aa changed the localization to the ER (Fig. 3D). Deletion of aa 75-94 similarly resulted in ER localization (Fig. 3E). We conclude that these 20 aa are required for HCN1 to exit from the ER and target to the ISPM.

Trafficking motifs are usually well conserved so we examined an alignment of the critical 20 amino acid region in HCN1 from multiple species and found that it is almost identical among amphibians, birds, and mammals (Fig. 3F). Therefore any or all of these residues could participate in trafficking. The first serine residue (Fig. 3F, arrow) in this region corresponds to a mutation (S100F) causing early infantile epileptic encephalopathy.[24] Human $HCN1_{S100F}$ has different physiological characteristics from the wildtype HCN1 and there is decreased surface expression of this mutant, indicating that the serine residue may be crucial for trafficking HCN1 to the plasma membrane. To test this, we expressed GFP-$HCN1_{S75F}$ (*X. tropicalis* equivalent of human S100F). We found that $HCN1_{S75F}$ is able to target to the ISPM with no more striking localization to internal membranes than the wild-type channel (Fig. 3G). We conclude that this serine is not essential for trafficking and the alterations to the biophysical properties of HCN1 are the most likely explanation for the development of epilepsy.

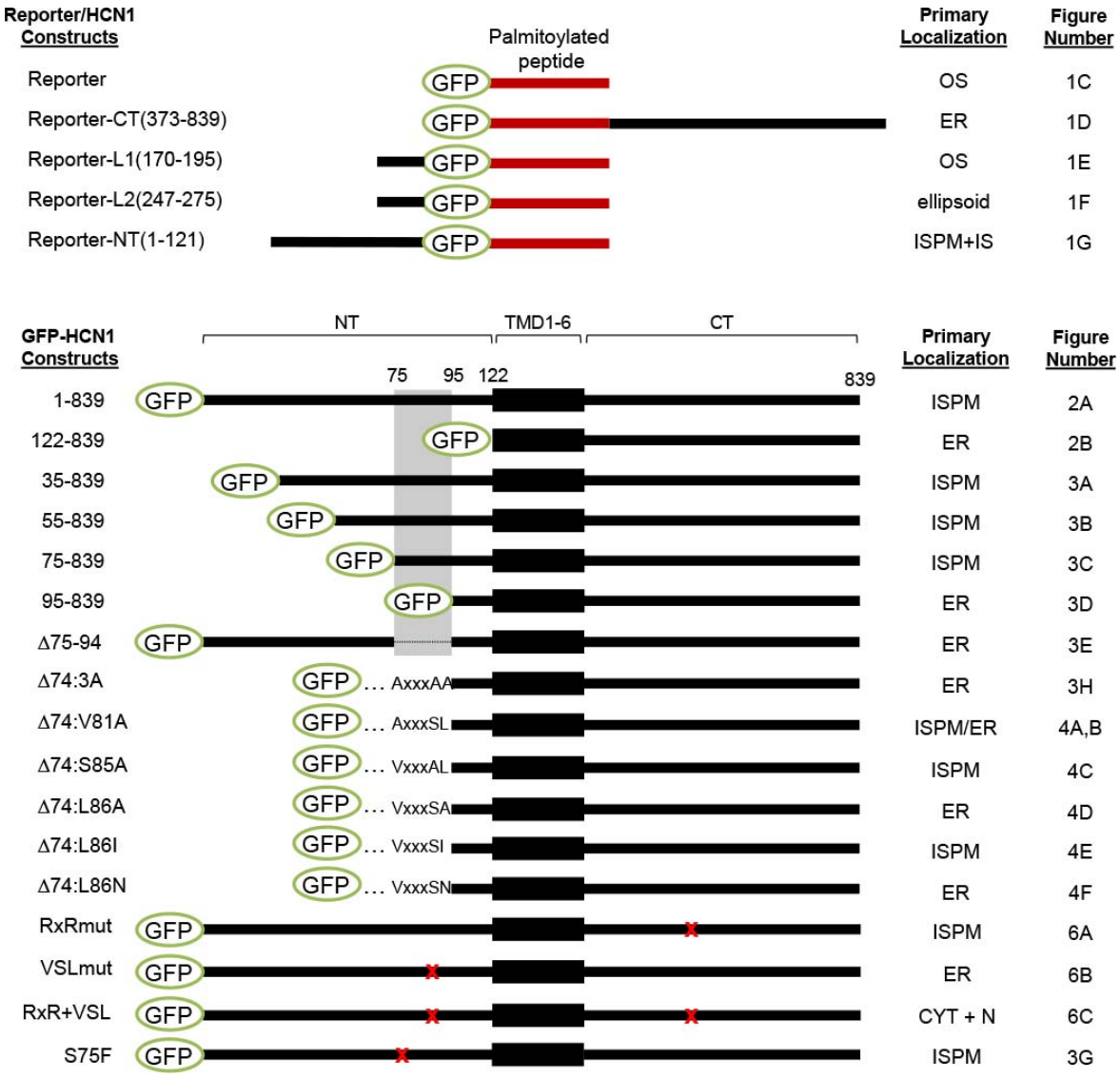
We next focused on investigating the –VNKFSL– sequence in the center of the trafficking motif because it is similar to a VxxSL ER export motif identified in another member of the voltage-gated potassium channel superfamily, Kv1.4.[25] Mutating any one of the three key residues decreased the amount of Kv1.4 present at the cell surface when tested in HEK293 cells. To determine if the VNKFSL sequence in HCN1 functions similarly, we mutated three of the residues to alanines (V81A, S85A, L86A; GFP-$HCN1\Delta_{74:3A}$). In support of the hypothesis, GFP-$HCN1\Delta_{74:3A}$ lost the ability to target the ISPM and instead was retained in the ER (Fig. 3H).

We tested the requirement for each of the three defining aa in the putative VxxxSL motif by mutating each to alanine (Fig. 4). Mutating the valine gave rise to a variable phenotype. In two of the six transgenic animals expressing GFP-$HCN1\Delta_{74:V81A}$ the channel localized primarily to the ISPM. But in the remaining four animals the channel localized to the ER. This variability likely reflects different expression levels in the individual animals and indicates that V81 is part of the trafficking motif but is not essential (Figs. 4A, 4B). Unlike the case with Kv1.4, the serine in the HCN1 trafficking motif is not required as GFP-$HCN1\Delta_{74:S85A}$ localized to the ISPM (Fig. 4C). Mutating the leucine abolished the protein's ability to target the ISPM as GFP-$HCN1\Delta_{74:L86A}$ invariantly localized to the ER (Fig. 4D). We conclude that L86 is key to the function of this trafficking motif and it functions more reliably with a valine several residues upstream.

To gain further insight into the properties needed for a functional ER export signal, we made two additional mutants. One mutant maintains the hydrophobicity and size of the residue by converting it to an isoleucine (GFP-$HCN1\Delta_{74:L86I}$). The other changes the valine to an asparagine thereby maintaining the size but not the hydrophobicity (GFP-$HCN1\Delta_{74:L86N}$). GFP-$HCN1\Delta_{74:L86I}$ localized to the ISPM while GFP-$HCN1\Delta_{74:L86N}$ was retained in the ER like GFP-$HCN1\Delta_{74:L86A}$, verifying that a relatively large hydrophobic residue is required at this position in the ER export motif (Figs. 4E, 4F).

It is possible that the trafficking defect we observe with the L86 mutations is secondary to a defect in overall protein folding and assembly. Sedimentation through 5% to 20% continuous sucrose gradients was used to test the assembly

Table. Summary of All Transgenic Constructs Presented in This Study



A summary of the design and targeting behavior of all constructs expressed in transgenic *X.laevis* photoreceptors. The reporter-HCN1 fusion constructs are listed on the top. The range of HCN1 amino acids attached to the reporter in each construct is listed in the first column. The GFP-HCN1 constructs are listed on the *bottom*. Regions of HCN1 are indicated above the *brackets*. The *gray shaded area* is required for ISPM localization. The primary localization and corresponding figure number for each construct are listed in the last two columns. TMD, transmembrane domain; L1/L2, intracellular loop 1/loop 2; CYT, cytoplasm.

state of exogenously expressed HCN1 channels. The distribution of HA-HCN1$_{WT}$ and HA-HCN1$_{VSLmut}$ were indistinguishable (Fig. 5). We used the sodium/potassium ATPase (NKA) as our internal size standard because the NKA$\alpha\beta$ heterodimer is approximately 130 kDa in size, similar to an HCN1 monomer. The distribution of NKA peaked much higher in the gradient than HCN1 at fraction 7 to 8 versus fraction 4. We conclude that under these conditions HCN1 is more likely an intact homotetramer than an unassembled monomer. We also generated the mutant with only the leucine in the VSL motif mutated. As expected, HA-HCN1$_{L100A}$ behaved the same as HA-HCN1$_{VSLmut}$ (Supplementary Fig. S2). This leads us to conclude that the mutation in this leucine (L)-based motif is primarily a trafficking defect.

The ER Export Motif Overrides the Function of the ER Retention Motif

In the next set of experiments we investigated the interaction between trafficking signals carried out by the leucine-based ER export motif and the di-arginine ER retention motif that we previously described.[15] The plasma membrane localization of both wild-type HCN1 and HCN1 with the di-arginine motif mutated (GFP-HCN1$_{RxRmut}$; Figs. 2A, 6A) indicates that the

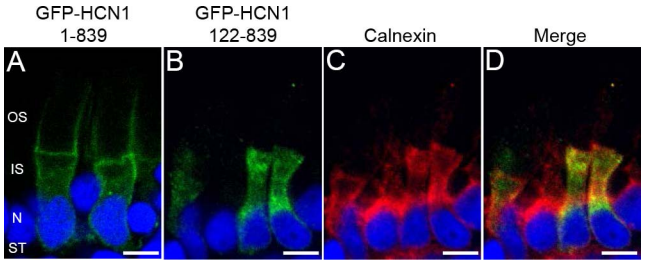


Figure 2. The NT is required for HCN1 to target the ISPM. Transgenic *X. laevis* rod photoreceptors expressing the GFP tagged (*green*) full-length HCN1 (**A**) or HCN1 mutant with the NT truncated (**B**) are shown. The section in (**B**) was also labeled with antibodies against calnexin ([**C**, **D**]; *magenta*). *Scale bars*: 5 μm.

leucine-based ER export signal dominates over the ER retention signal. Alternatively, there could be additional as yet unknown motifs promoting ER exit. To test this, we used the triple mutant of the leucine-based ER export motif described above (VNKFSL to ANKFAA) in the context of full-length HCN1, with an intact di-arginine ER retention motif (GFP-HCN1$_{VSLmut}$, Fig. 6B). This mutant localized to the ER in *X. laevis* photoreceptors, leading us to conclude that if there are any additional ER export motifs in this channel they are not strong enough on their own to counteract the information conveyed by the di-arginine ER retention motif. Curiously, when we incorporated both mutations (GFP-HCN1$_{RxR\ +\ VSL}$) the GFP signal was found diffusely throughout the photoreceptor OS and IS, including within the nucleus (Fig. 6C). This pattern could indicate partial ER localization but is more characteristic of the pattern observed when soluble GFP is expressed.[22] The most likely explanation for how the GFP-tagged HCN1 double mutant could have a soluble localization pattern is if the protein is partially degraded. This idea is consistent with the known phenomenon that mistrafficked proteins are degraded.[26]

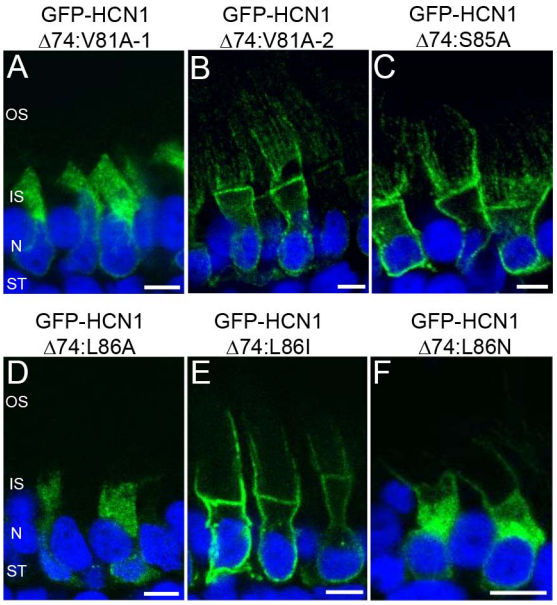


Figure 4. A large hydrophobic residue is needed in the ER export signal. (**A**–**F**) Transgenic *X. laevis* rod photoreceptors expressing the indicated GFP-HCN1 mutants detailed in Table. Note, two images of the GFP-HCN1$\Delta_{74:V81A}$ mutation are shown to demonstrate the range of phenotypes observed with this mutant (**A**, **B**). *Scale bars*: 5 μm.

Altogether, our results point to the importance of both ER transport signals in maintaining proper expression levels of HCN1 with the leucine-based ER export motif dominating over the di-arginine ER retention motif.

Discussion

HCN1 channels modulate multiple aspects of neuronal function. HCN1 is subject to multiple levels of regulation,

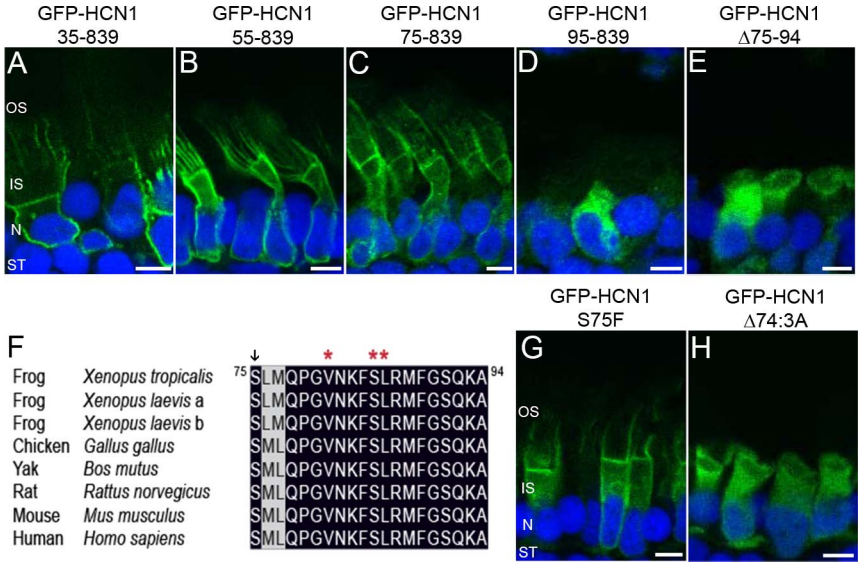


Figure 3. Identification of a VxxxSL ER export motif in HCN1. Transgenic *X, laevis* rod photoreceptors expressing the indicated GFP-HCN1 truncation (**A**–**E**) and point mutation (**G**, **H**) mutants detailed in Table are shown. (**F**) ClustalW sequence alignment of *X. tropicalis* HCN1 (aa75-94) to HCN1 from various animal species. Identical residues shaded in *black*, partially conserved residues in *gray*. The VxxxSL motif is marked with *red asterisks*. The serine residue found to be mutated in epilepsy patients is indicated by the *black arrow*. Accession numbers are: *X. tropicalis*, XP002933077; *X. laevis* a, b deduced from genomic scaffold v7.1 52441 and 337825; *G. gallus*, XP429145; *B. mutus*, XP005909291; *R. norvegicus*, W9JKB0; *M. musculus*, O88704; *H. sapiens*, O60741. *Scale bars*: 5 μm.

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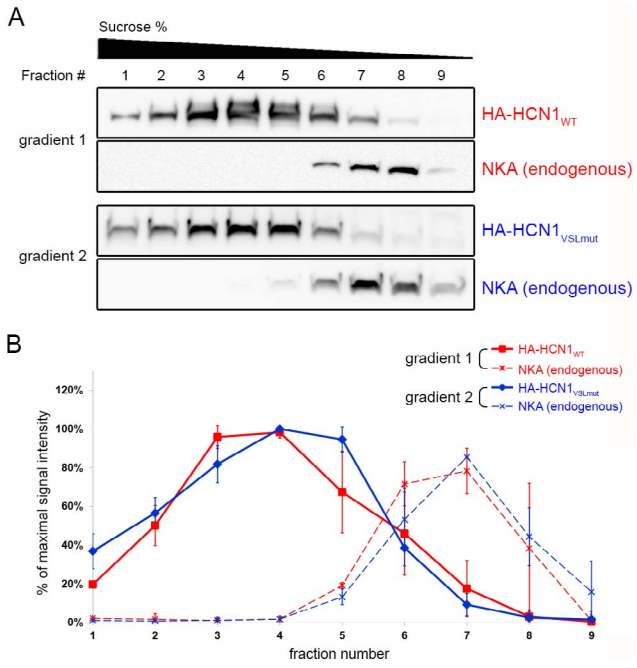

Figure 5. Disrupting the leucine-based ER export motif does not change the assembly of HCN1 channels. (**A**) Lysates from the HEK293 cells expressing either HA-tagged wild-type ($HCN1_{WT}$) HCN1 or mutant HCN1 with the ER export motif mutated ($HCN1_{VSLmut}$) were subjected to velocity sedimentation through 5% to 20% sucrose gradients. Fractions collected from the sucrose gradient were analyzed via Western blotting. The endogenous sodium potassium ATPase (NKA) was used as the standard for protein size and fraction alignment. (**B**) Quantification of the chemiluminescent signals from (**A**) and no statistically significant difference was detected between HA-$HCN1_{WT}$ and HA-$HCN1_{VSLmut}$.

including controlling the amount of HCN1 at the plasma membrane by integrating information from multiple trafficking signals. We previously reported the discovery of a di-arginine ER retention signal, which is a negative regulator of HCN1 surface expression.[15] The aim of this study was to identify a forward trafficking signal that could counter the action of the negative regulator thereby increasing surface expression. By expressing a series of HCN1 mutants in transgenic *X. laevis* photoreceptors, we identified a leucine-based ER export signal that positively regulates the plasma membrane targeting of HCN1.

The ER export signal in HCN1 is located in the cytoplasmic NT near the first transmembrane domain. This region is highly conserved, both across species and even among the related HCN channels, indicating a shared mechanism of channel trafficking. $HCN1_{L86}$ is essential to the function of the ER export signal, as mutation of this residue to either alanine or asparagine resulted in ER retention of the channel. The critical leucine in HCN1 is preceded by a valine and serine (VxxxSL) in a pattern similar to the ER export signal found in Kv1.4 (VxxSL).[27] However, the order of importance of the three residues is different; in Kv1.4 it is L > S > V, but in HCN1 the order is L > V with S being dispensable. Our observation, and the study on Kv1.4, point out the importance of the large hydrophobic leucine residue in facilitating ER export. It is likely that the ER export signals in HCN1 and Kv1.4 function in the same manner but the importance of individual residues varies based on their microenvironment.

Endoplasmic reticulum export signals function by recruiting COPII coat components, which then polymerize and deform the membrane so that cargo-containing transport

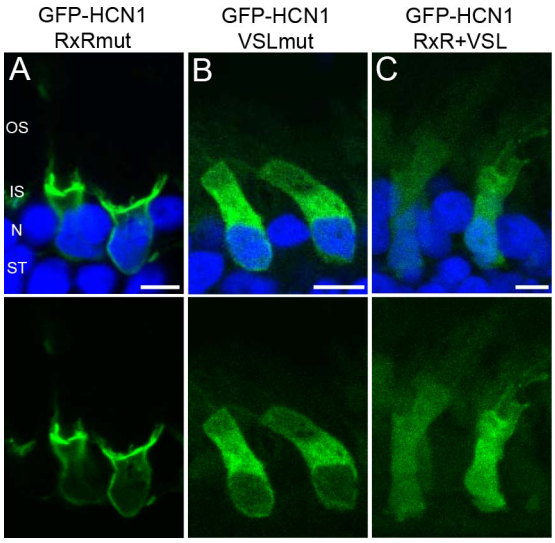

Figure 6. The leucine-based ER export motif counteracts the function of the ER retention signal (**A**) GFP-HCN1 with an intact ER export signal but mutated ER retention signal accumulates at the ISPM in transgenic *X. laevis* rod photoreceptors (GFP-$HCN1_{RxRmut}$). (**B**) Mutating the ER export signal alone prevents accumulation at the ISPM (GFP- $HCN1_{VSLmut}$). (**C**) Mutation of both trafficking signals leads to diffuse distribution of GFP throughout the cytoplasm and nucleoplasm. *Lower panels* are the same image as in the *upper panels* with the nuclear stain not shown. *Scale bars*: 5 μm.

vesicles bud off from the ER.[28] The primary cargo receptor that binds to ER export signals is Sec24. While not yet established, a likely mechanism for the HCN1 ER export motif is to recruit Sec24 either directly or indirectly. Sec 24 has the remarkable ability to select a diverse array of cargo proteins in part because it has multiple cargo binding sites; for instance, Sec24 binds NPF motifs at the A site and DxE or LxxLE motifs at the B site.[29–31] A very intriguing recent study discovered that many multipass integral membrane proteins can bind directly to Sec24 and indirectly via the Erv14 receptor.[32] The authors propose that cargo selection by coincidence detection is likely to enhance the efficiency of selectively sorting proteins destined for the plasma membrane. The leucine-based motif we have identified in HCN1 is most similar to hydrophobic motifs (e.g., the IRFTL motif in Erv14[33]). Elucidation of the mechanisms of HCN1 and COPII recruitment would be helpful in further delineating the trafficking pathway of HCN1.

Trafficking out of the ER is a major quality control checkpoint. Properly folded and, in the case of multisubunit complexes, assembled cargo proteins should be the most efficient at recruiting Sec24. It is not completely understood how this occurs. In some cases oligomerization in and of itself may be the signal for export.[34] In the case of GPI-anchored luminal cargoes, maturation of the glycan moiety is required for binding the p24 receptor, which then recruits Lst1p (a Sec24 homologue).[35] The velocity sedimentation profile of HCN1, which takes into account the size, shape, and density of multisubunit complexes, was not changed when the leucine-based ER export motif was mutated therefore this motif is not needed for oligomerization of the channel. Perhaps either the glycosylation of HCN1 and/or oligomerization promotes binding to a sorting receptor similar to p24 or Erv14.

The necessity of properly regulating HCN1 is illustrated by de novo HCN1 mutations found in patients with early infantile epileptic encephalopathy.[24] The $HCN1_{S100F}$ mutation has decreased surface expression in CHO cells, implying a possible trafficking defect. The S100 residue is in close proximity to the

ER export signal therefore may affect its function. Alternatively, the S100F mutation may alter the stability of HCN1 because the mutant also displays high susceptibility to proteasomal degradation. We tested the effect of the S100F mutation in HCN1 trafficking/stability in vivo in *X. laevis* photoreceptors, but the mutant does not show obvious trafficking or stability defects in this system. It should be noted that photoreceptors seem to have a greater tolerance for HCN1 mutation/degradation than cortical and hippocampal neurons given that TRIP8b knockout mice with approximately 40% loss of functional HCN1 retain normal vision but have defects in motor learning and greater resistance to depressive behaviors.[6] Transgenic mice expressing disease-specific HCN1 mutants at a physiological level will help to elucidate the cell-type specific requirements for HCN1 trafficking in the progression of disease.

A challenge remaining for future studies is to pinpoint the signals involved in targeting HCN1 to the IS compartment of the plasma membrane in photoreceptors. Exogenously expressed HCN1 localizes to the ISPM and is excluded from the OS, consistent with the localization of endogenous HCN1 as determined by immunostaining studies in mammalian species and electrophysiological recordings in multiple species.[3,11,36–40] It was shown previously in *X. laevis* photoreceptors that exogenously expressed membrane proteins require some type of IS targeting information to escape the bulk flow of membrane to the continuously renewing OS.[41] The IS compartmentalization signal of HCN1 is unlikely to be present in the C-terminus because this portion of the channel can only redirect reporters to the ER.[15] Furthermore, reporter constructs with mutations in the ER retention signal are released to the OS, not to the ISPM. The NT of HCN1 may contain at least part of the IS targeting information because this portion of the channel has the ability to redirect the reporter to the ISPM, though some of these proteins are also found to variable degrees within internal compartments. Our truncation mutants of GFP-HCN1 suggests that such a signal may reside in the essential 20 amino acid region we mapped in this study (aa75-aa94 in *Xenopus tropicalis* HCN1) because GFP-HCN1$_{75\text{-}839}$ was able to target the ISPM. However, further truncation mutants are not favorable for identification of the IS compartmentalization signal because any mutation that cripples the ER export signal allowed the ER retention signal to predominate such that the channels were trapped at the earliest stage of trafficking through the endomembrane system. Site-specific mutations across aa75-121 of HCN1 without affecting the ER export signal will be required to test this idea.

In conclusion, we identified a novel leucine-based hydrophobic ER export signal. The signal serves as a positive regulator in controlling the surface amount of HCN1 by promoting trafficking of HCN1 from the ER to the surface. There are two major trafficking signals controlling the equilibrium between internal and surface pools of HCN1 in photoreceptors. Under normal conditions, the ER export signal dominates and the majority of the channels are found in the plasma membrane of the IS. Crippling the di-arginine ER retention signal releases more HCN1 so that the surface levels increase. Crippling the ER export signal allows the ER retention signal to dominate. When both signals are disrupted the protein is likely subject to degradation. Overall, this work reveals a hierarchy of trafficking signals regulating the surface availability of HCN1 channels.

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References

1. Wahl-Schott C, Biel M. HCN channels: structure, cellular regulation and physiological function. *Cell Mol Life Sci*. 2009; 66:470–494.
2. Barrow AJ, Wu SM. Low-conductance HCN1 ion channels augment the frequency response of rod and cone photoreceptors. *J Neurosci*. 2009;29:5841–5853.
3. Knop GC, Seeliger MW, Thiel F, et al. Light responses in the mouse retina are prolonged upon targeted deletion of the HCN1 channel gene. *Eur J Neurosci*. 2008;28:2221–2230.
4. Seeliger MW, Brombas A, Weiler R, et al. Modulation of rod photoreceptor output by HCN1 channels is essential for regular mesopic cone vision. *Nat Commun*. 2011;2:532.
5. Shin M, Brager D, Jaramillo TC, Johnston D, Chetkovich DM. Mislocalization of h channel subunits underlies h channelopathy in temporal lobe epilepsy. *Neurobiol Dis*. 2008;32:26–36.
6. Lewis AS, Vaidya SP, Blaiss CA, et al. Deletion of the hyperpolarization-activated cyclic nucleotide-gated channel auxiliary subunit TRIP8b impairs hippocampal Ih localization and function and promotes antidepressant behavior in mice. *J Neurosci*. 2011;31:7424–7440.
7. Santoro B, Hu L, Liu H, et al. TRIP8b regulates HCN1 channel trafficking and gating through two distinct C-terminal interaction sites. *J Neurosci*. 2011;31:4074–4086.
8. Piskorowski R, Santoro B, Siegelbaum SA. TRIP8b splice forms act in concert to regulate the localization and expression of HCN1 channels in CA1 pyramidal neurons. *Neuron*. 2011;70: 495–509.
9. Han Y, Noam Y, Lewis AS, et al. Trafficking and gating of hyperpolarization-activated cyclic nucleotide-gated channels are regulated by interaction with tetratricopeptide repeat-containing Rab8b-interacting protein (TRIP8b) and cyclic AMP at distinct sites. *J Biol Chem*. 2011;286:20823–20834.
10. Wilkars W, Liu Z, Lewis AS, et al. Regulation of axonal HCN1 trafficking in perforant path involves expression of specific TRIP8b isoforms. *PLoS One*. 2012;7:e32181.
11. Pan Y, Bhattarai S, Modestou M, Drack AV, Chetkovich DM, Baker SA. TRIP8b is required for maximal expression of HCN1 in the mouse retina. *PLoS One*. 2014;9:e85850.
12. Akhavan A, Atanasiu R, Noguchi T, Han W, Holder N, Shrier A. Identification of the cyclic-nucleotide-binding domain as a conserved determinant of ion-channel cell-surface localization. *J Cell Sci*. 2005;118:2803–2812.
13. Gravante B, Barbuti A, Milanesi R, Zappi I, Viscomi C, DiFrancesco D. Interaction of the pacemaker channel HCN1 with filamin A. *J Biol Chem*. 2004;279:43847–43853.
14. Noam Y, Ehrengruber MU, Koh A, et al. Filamin A promotes dynamin-dependent internalization of hyperpolarization-activated cyclic nucleotide-gated type 1 (HCN1) channels and restricts Ih in hippocampal neurons. *J Biol Chem*. 2014;289: 5889–5903.
15. Pan Y, Laird JG, Yamaguchi DM, Baker SA. A di-arginine ER retention signal regulates trafficking of HCN1 channels from the early secretory pathway to the plasma membrane. *Cell Mol Life Sci*. 2015;72:833–843.
16. Ramakrishnan NA, Drescher MJ, Khan KM, Hatfield JS, Drescher DG. HCN1 and HCN2 are expressed in cochlear hair cells: HCN1 can form a ternary complex with protocadherin 15 CD3 and F-actin-binding filamin A or can interact with HCN2. *J Biol Chem*. 2012;287:37628–37646.
17. Reiners J, Marker T, Jurgens K, Reidel B, Wolfrum U. Photoreceptor expression of the Usher syndrome type 1 protein protocadherin 15 (USH1F) and its interaction with the

scaffold protein harmonin (USH1C). *Mol Vis*. 2005;11:347–355.

18. Sahly I, Dufour E, Schietroma C, et al. Localization of Usher 1 proteins to the photoreceptor calyceal processes, which are absent from mice. *J Cell Biol*. 2012;199:381–399.
19. Burns ME, Arshavsky VY. Beyond counting photons: trials and trends in vertebrate visual transduction. *Neuron*. 2005;48: 387–401.
20. Amaya E, Kroll KL. A method for generating transgenic frog embryos. *Methods Mol Biol*. 1999;97:393–414.
21. Kroll KL, Amaya E. Transgenic Xenopus embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development*. 1996;122:3173–3183.
22. Tam BM, Moritz OL, Hurd LB, Papermaster DS. Identification of an outer segment targeting signal in the COOH terminus of rhodopsin using transgenic Xenopus laevis. *J Cell Biol*. 2000; 151:1369–1380.
23. Salinas RY, Baker SA, Gospe SM III, Arshavsky VY. A single valine residue plays an essential role in peripherin/rds targeting to photoreceptor outer segments. *PLoS One*. 2013; 8:e54292.
24. Nava C, Dalle C, Rastetter A, et al. De novo mutations in HCN1 cause early infantile epileptic encephalopathy. *Nat Genet*. 2014;46:640–645.
25. Li D, Takimoto K, Levitan ES. Surface expression of Kv1 channels is governed by a C-terminal motif. *J Biol Chem*. 2000; 275:11597–11602.
26. Young JC. The role of the cytosolic HSP70 chaperone system in diseases caused by misfolding and aberrant trafficking of ion channels. *Dis Model Mech*. 2014;7:319–329.
27. Zhu J, Watanabe I, Gomez B, Thornhill WB. Trafficking of Kv1.4 potassium channels: interdependence of a pore region determinant and a cytoplasmic C-terminal VXXSL determinant in regulating cell-surface trafficking. *Biochem J*. 2003;375: 761–768.
28. D'Arcangelo JG, Stahmer KR, Miller EA. Vesicle-mediated export from the ER: COPII coat function and regulation. *Biochim Biophys Acta*. 2013;1833:2464–2472.
29. Miller E, Antonny B, Hamamoto S, Schekman R. Cargo selection into COPII vesicles is driven by the Sec24p subunit. *EMBO J*. 2002;21:6105–6113.
30. Miller EA, Beilharz TH, Malkus PN, et al. Multiple cargo binding sites on the COPII subunit Sec24p ensure capture of diverse membrane proteins into transport vesicles. *Cell*. 2003; 114:497–509.
31. Mossessova E, Bickford LC, Goldberg J. SNARE selectivity of the COPII coat. *Cell*. 2003;114:483–495.
32. Pagant S, Wu A, Edwards S, Diehl F, Miller EA. Sec24 is a coincidence detector that simultaneously binds two signals to drive ER export. *Curr Biol*. 2015;25:403–412.
33. Powers J, Barlowe C. Erv14p directs a transmembrane secretory protein into COPII-coated transport vesicles. *Mol Biol Cell*. 2002;13:880–891.
34. Springer S, Malkus P, Borchert B, Wellbrock U, Duden R, Schekman R. Regulated oligomerization induces uptake of a membrane protein into COPII vesicles independent of its cytosolic tail. *Traffic*. 2014;15:531–545.
35. Manzano-Lopez J, Perez-Linero AM, Aguilera-Romero A, et al. COPII coat composition is actively regulated by luminal cargo maturation. *Curr Biol*. 2015;25:152–162.
36. Muller F, Scholten A, Ivanova E, Haverkamp S, Kremmer E, Kaupp UB. HCN channels are expressed differentially in retinal bipolar cells and concentrated at synaptic terminals. *Eur J Neurosci*. 2003;17:2084–2096.
37. Demontis GC, Moroni A, Gravante B, et al. Functional characterisation and subcellular localisation of HCN1 channels in rabbit retinal rod photoreceptors. *J Physiol*. 2002;542:89–97.
38. Fyk-Kolodziej B, Pourcho RG. Differential distribution of hyperpolarization-activated and cyclic nucleotide-gated channels in cone bipolar cells of the rat retina. *J Comp Neurol*. 2007;501:891–903.
39. Akopian A, Witkovsky P. D2 dopamine receptor-mediated inhibition of a hyperpolarization-activated current in rod photoreceptors. *J Neurophysiol*. 1996;76:1828–1835.
40. Fain GL, Quandt FN, Bastian BL, Gerschenfeld HM. Contribution of a caesium-sensitive conductance increase to the rod photoresponse. *Nature*. 1978;272:466–469.
41. Baker SA, Haeri M, Yoo P, et al. The outer segment serves as a default destination for the trafficking of membrane proteins in photoreceptors. *J Cell Biol*. 2008;183:485–498.

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