Mechanisms of HCN channel trafficking and surface expression
Noam, Y.Y.

Citation for published version (APA):
Noam, Y. Y. (2011). Mechanisms of HCN channel trafficking and surface expression

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Subcellular targeting and surface expression of HCN1 channels via the actin-binding protein filamin A

Yoav Noam, Lise Phan, Markus U. Ehrengruber, Paul Feyen, Erik M. Manders, Tallie Z. Baram & Wytse J. Wadman

In preparation
Abstract

Ion channel trafficking and membrane dynamics are critical to neuronal function and plasticity. Among the ion channels that govern the electric properties of neurons, hyperpolarization-activated cyclic nucleotide-gated (HCN) channels play versatile roles in the fine-tuning of neuronal excitability. Filamin A (FLNa) is a cytoskeletal protein that in addition to its actin-stabilizing roles, can bind to ion channels, including HCN1 channels, influencing their trafficking and surface expression. We explored cellular mechanisms that underlie HCN1 channel regulation by FLNa and the functional consequences of HCN1-FLNa interactions. Co-expression of fluorophore-fused HCN1 and FLNa cDNA constructs in HEK293 cells resulted in sequestration of HCN1 channels, evident by the accumulation of HCN1 in intracellular clusters. This FLNa-induced clustering of HCN1 was abrogated when HCN1-FLNa or FLNa-actin interactions were disrupted, using either an HCN1 channel construct lacking the FLNa-binding domain or a FLNa construct lacking the actin-binding domains. FLNa-induced clustering of HCN1 was associated with altered surface distribution of the channel and reduced $I_h$, assessed using total internal reflection fluorescent microscopy (TIRF-M) and whole-cell voltage clamp measurements, respectively. Additionally, FLNa increased the presence of HCN1 channel protein in endosomal organelles, and particularly within lysosomes. Both the HCN1 clustering and $I_h$ down-regulation were reversed by selective inhibition of the GTPase dynamin, indicating a role for active internalization in FLNa-mediated HCN1 regulation. Taken together, these data support an inhibitory role for FLNa on HCN1 channel surface expression and function, mediated by dynamin-dependent internalization and endosomal targeting of the channel.
Introduction

Trafficking and surface expression of ion channels are highly regulated mechanisms that help shape the excitable properties of the membrane. The exquisite control of ion channel insertion and lateral mobility within the cell membrane, as well as of internalization into the cytoplasm underlies many forms of neuronal plasticity (Shah et al., 2010), and failure of these mechanisms may result in pathological conditions such as epilepsy (Beck and Yaari, 2008).

Among the myriad ion channels that control neuronal excitability, HCN channels are an important class of voltage-gated channels that mediate the hyperpolarization-activated conductance $I_h$. Active at sub-threshold potentials, $I_h$ plays versatile roles in the regulation of neuronal excitability throughout the brain (Santoro and Baram, 2003; Biel et al., 2009). Four HCN channel isoforms (HCN1-4) exist, contributing to the diversity of $I_h$ properties across tissues, cell types and subcellular domains (Biel et al., 2009). Rather than providing a tonic, background conductance to the cell, $I_h$ is a dynamically regulated current that responds to changes in the physiological milieu: alterations in neuronal activity may result in either up- or down-regulation of $I_h$ amplitude at multiple time frames (Noam et al., 2011), and this dynamic behavior of $I_h$ is involved in various forms of plasticity (van Welie et al., 2004; Fan et al., 2005; Brager and Johnston, 2007; Campanac et al., 2008; Noam et al., 2010; Narayanan et al., 2010).

HCN channel trafficking is emerging as a key mechanism for $I_h$ regulation: selective distribution of HCN channel isoforms to distinct sub-cellular compartments and during development has been reported in several types of neuronal populations (Lorincz et al., 2002; Brewster et al., 2007; Bender et al., 2007; Huang et al., 2011), and mechanisms of HCN channel trafficking and surface expression may be activity-dependent (Shin and Chetkovich, 2007; Noam et al., 2010). Whereas the mechanisms that promote selective distribution and activity-dependent trafficking of HCN channels remain largely unknown, the discovery of several auxiliary proteins that interact with HCN channels may shed light on these processes (Lewis et al., 2010). Among these candidates, filamin A (FLNa) is an actin-binding protein that can directly interact with HCN1 channels, but does not interact with either HCN2 or HCN4 channels (Gravante et al., 2004).

FLNa is a cytoskeletal protein that stabilizes the actin network by crosslinking actin filaments in a perpendicular orientation (Stossel et al. 2001; Nakamura et al. 2007). This function promotes the formation of a stable, actin lattice-type structure. In addition to its structural roles, FLNa interacts with many membrane proteins, including several ion channels, and
Regulates their surface expression and trafficking (Petrecca et al., 2000; Lin et al., 2001; Onoprishvili et al., 2003; Kim et al., 2007b). Interaction of HCN1 channels with FLNa occurs via a 22 amino acid sequence at the C’ terminus of the channel (Gravante et al., 2004). Knockout of FLNa augments I_h amplitude and facilitates channel gating (by speeding activation kinetics and shifting the voltage-activation profile to more depolarized values), suggesting an inhibitory role for FLNa on HCN1 channels (Gravante et al., 2004). In addition to these functional effects, FLNa knockout influences surface expression of the channel: HCN1 channels are homogenously distributed on the surface of FLNa-depleted melanoma cell lines (M2), whereas a non-uniform membrane distribution of HCN1 is observed on the membrane of FLNa-expressing (A7) cell lines (Gravante et al., 2004). Considering the roles of FLNa in regulation of ion channel trafficking and surface expression, we sought to examine the effects of FLNa on HCN channels. Employing a mutational approach to selectively interfere with HCN1-FLN interaction in HEK239 cells, we explored the cellular mechanisms by which FLNa regulates HCN channels, and their physiological implications.

Experimental Procedures

Plasmid cDNA constructs
A plasmid cDNA construct containing the EGFP sequence at the carboxy terminus of the mouse HCN1 DNA (inserted between aa 885 and 886, see Lewis et al., 2009; Noam et al., 2010) was a gift from Dr Chetkovich (Northwestern University). N’ terminus, EGFP-fused mouse HCN1 and HCN2 cDNA constructs (HCN1_{GFP,N’} and HCN2_{GFP}) were a gift from Drs Santoro and Siegelbaum (Columbia University); A point mutation (G44R) in the original HCN1GFP-N’ sequence, which originates from an earlier sequencing version of the mouse gene (NCBI NM_01048.1), was corrected for by PCR reaction (Genscript) to yield the correct mouse HCN1 sequence (NCBI NM_010408.3). A HCN1_{GFP} channel construct that lacks the filamin binding domain (HCN1_{Δ22,GFP}) was generated by deleting the 22 aa sequence in the mouse HCN1 C’ terminus that was previously reported to form the interaction domain (Gravante et al. 2004): 694-SPPIQSLATRTFHYASPTASQ-715. The final plasmid construct was confirmed by sequencing and yielded functional I_h upon expression in HEK293 cells (Sup. Fig. 1). Human FLNa with N-terminal fusion of monomeric DsRed (FLNa_{DsRed}) was a gift from Dr Nakamura (Harvard Medical School, MA, USA). Expression of FLNa_{DsRed} in HEK293 cells resulted in filamentous-like distribution pattern that resembled the distribution of endogenous FLNa in these cells (Sup. Fig. 2). The FLNa_{DsRed} construct was used as a
Chapter 5

Template to isolate IgG-like domains 23 and 24 of FLNa by PCR with the following primers:

5’-GTGCTCGAGGGGACCCAGGCTTGGTGTC-3’ (FLNa IgG23 fwd; possessing a XhoI site before the start of IgG-like domain 23);

5’-CTTCAATTGAATTCAAGGGCACCACAACGCGG-3’ (FLNa IgG24 rev; containing an EcoRI site at the regular filamin A stop codon, followed by an MfeI site).

The corresponding PCR fragment was restricted with XhoI and MfeI and inserted into FLNaDsRed construct that had been previously cut with XhoI and MfeI. This resulted in plasmid FLNa[23-24]DsRed where monomeric DsRed (225 aa) is coupled via a two amino acid linker (Ser-Arg) to the C-terminal 220 amino acids of human FLNa (encompassing IgG-like domains 23-24).

The description of all constructs used in this study, and their nomenclature, is summarized in Table 1.

Table 1: DNA constructs used in this study

<table>
<thead>
<tr>
<th>Construct name</th>
<th>Description</th>
<th>Fluorophore fusion site</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCNI_{GFP}</td>
<td>EGFP-fused, full-length mouse HCN1</td>
<td>C’ terminus</td>
</tr>
<tr>
<td>HCNI_{GFP,N'}</td>
<td>EGFP-fused, full-length mouse HCN1</td>
<td>N’ terminus</td>
</tr>
<tr>
<td>HCNI[Δ22]_{GFP}</td>
<td>EGFP-fused, mouse HCN1 sequence lacking the 22 amino acids that constitute the FLNa-binding domain (aa 694-715)</td>
<td>C’ terminus</td>
</tr>
<tr>
<td>HCNI_{GFP}</td>
<td>EGFP-fused, full-length mouse HCN1</td>
<td>N’ terminus</td>
</tr>
<tr>
<td>FLNa_{DsRed}</td>
<td>Monomeric DsRed-fused, full-length human FLNa</td>
<td>N’ terminus</td>
</tr>
<tr>
<td>FLNa[23-24]_{DsRed}</td>
<td>Monomeric DsRed-fused, truncated human FLNa, including only the C’ terminus-located IgG-like domains 23 and 24 (consisting of the HCN1-binding region and dimerization domain, but lacking the actin-binding domains).</td>
<td>N’ terminus</td>
</tr>
</tbody>
</table>
Cell culture and transfection with plasmid DNA

Human Embryonic Kidney 293 (HEK293) cells were obtained from ATCC. Cultures were maintained in Minimum Essential Medium (MEM) supplemented with 100 μg/ml penicilline-streptomycine, 2 mM glutamine, and 10% fetal bovine serum. The cells were kept in a humidified atmosphere, at 37°C and 5% CO₂, were passaged weekly and refreshed every 2-3 days. All culture reagents were from Invitrogen. Transfection with cDNA constructs was performed using the calcium-phosphate precipitation method as previously described (Noam et al., 2008; Han et al., 2011). Briefly, 1-3 days prior to transfection, cells were re-plated on 12 mm glass coverslips at 50-60% density. For the expression of HCN channels and/or FLNa constructs, 0.3 microgram of plasmid cDNA was used per construct per coverslip, resulting in a 1:1 DNA ratio in co-transfection experiments. All experiments were performed 24-48 hours post-transfection.

Fixation and Immunocytochemistry

Fixation of transfected HEK293 cells for imaging purposes was performed by 15 minutes incubation with PBS solution + 4% paraformaldehyde (PFA) on ice. PFA was removed by a series of washes with PBS (5 x 5 minutes), and coverslips were mounted on a glass microscope slide using Fluoromount G mounting medium (Southern Biotech). Immunolabeling experiments with anti-FLNa or anti-EEA1 (an early endosomal marker) were performed by PFA fixation as described above followed by 10 minutes permeabilization with 0.1% Triton X-100, and 1 hour incubation with PBS + 5% normal goat serum + 1% BSA at room temperature, to block non-specific interactions. Primary antibodies were diluted in PBS + 1% BSA and were applied for 1 hour at room temperature. The antibody was removed by a series of washes (3 x 5 minutes with PBS) followed by incubation with a secondary, mouse alexa-633-conjugated- or mouse-alexa-488- antibody (Invitrogen) in PBS + 1% BSA solution. Secondary antibody was removed by a series of washes with PBS (3 x 5 minutes) and mounted on glass microscope slides as described above. To label lysosomes, LAMP1 staining was performed by fixing and permeabilizing the cells in ice-cold methanol, followed by blocking and antibody incubation as described above. Antibodies used in this study included the mouse monoclonal anti-LAMP1 (Abcam; clone [H4A3]; 1:500), mouse monoclonal anti-EEA1 (clone 13; BD Transduction Laboratories; 1:2000) and mouse monoclonal anti-FLNa (clone PM6/317; Millipore; 1:1000). All immunocytochemistry experiments were performed at least in duplicates and repeated at least at three separate occasions. Differences in subcellular distribution were assessed blindly.

Transferrin-uptake assay

To label recycling endosomes, HEK293 cells expressing HCN1<sub>GFP</sub> and FLNa<sub>DsRed</sub> were first
starved of transferrin by 15 minutes incubation with serum-deficient MEM medium, followed by 1 hour incubation with 50 μg/ml alexa-633–conjugated transferrin (Invitrogen), at 37°C. Cells were quickly rinsed with ice-cold PBS, and fixed with 4% PFA as described above.

**Dynamin inhibition**

Dynamin inhibition was carried out by incubating HEK293 cells with 80 μM of the selective dynamin inhibitor dynasore (Sigma) for a period of 3-4 hours, at 37°C. As a vehicle treatment, a similar amount of the organic solvent (0.26% DMSO) was applied, for a similar duration.

**Confocal Imaging**

Confocal imaging was performed using an LSM-510 confocal microscope (Zeiss) equipped with an Apochromat 63x oil objective (numeric aperture = 1.40). Samples that contained more than one fluorophore were scanned sequentially using the “multi-track” mode with separate excitation beams: an Argon laser at a wavelength of 488 nm for GFP imaging, a He/Ne laser at 543 nm for DsRed imaging, and a He/Ne laser beam at 633 nm for far-red imaging (of endosomal markers conjugated to alexa-633 fluorophore). Images were digitized at 12 bit, in a frame size of 1024 x 1024 pixel. Optical slices were scanned at a thickness of <1 μm. Final image adjustments were performed using ImageJ software (NIH): images were background-subtracted using the “BG subtraction from ROI” plugin (M. Cammer and T. Collins) and a mild median filter was applied (radius=0.2 pixels) for presentation purposes.

**Total Internal Reflection Fluorescence Microscopy (TIRF-M)**

TIRF-M was performed using an inverted Nikon Ti microscope with a TIRF attachment, equipped with an oil immersion 60x Apo TIRF objective (numeric aperture = 1.49). Fluorescent excitation was employed using an Argon/ Krypton laser at wavelengths of 488 nm and 568 nm for GFP and DsRed imaging, respectively. GFP and DsRed signals were selected using bandpass filters at wavelengths of 525/50 nm and 625/50 nm, respectively. Images were collected using an Andor iXon 897 CCD camera.

**Electrophysiology**

I_h was recorded from transfected HEK293 cells using the whole-cell voltage-clamp configuration as recently described (Han et al., 2011). The recording chamber was continuously perfused with an extracellular recording solution containing (in mM): 110 NaCl, 5 KCl, 2 CaCl_2, 1 MgCl_2, 0.5 cAMP, 10 HEPES, (pH set to 7.30). Recording pipettes (pulled from borosilicate glass) had a resistance of 2-5 MΩ when filled with an intracellular solution containing (in mM) 105 K-gluconate, 30 KCl, 2 Mg-ATP, 5 EGTA, 0.5 CaCl_2, 10
HEPES (pH set to 7.30). Series resistance was compensated for at least 75%. Only traces with voltage errors smaller than 2 mV were considered for analysis. Currents were acquired with an Axopatch 200B amplifier (Molecular Devices), controlled by a custom-written program in MATLAB (Mathworks). Currents were low-pass Bessel filtered at 2 kHz and sampled at 5 kHz. Liquid junction potential (calculated at 13 mV) was corrected for offline.

$I_h$ was evoked by a series of hyperpolarizing steps from a resting membrane potential of -50 mV to -130 mV, in decrements of 10 mV. $I_h$ amplitude was calculated by subtracting the steady-state current at the end of the voltage step from the instantaneous current at its beginning, as previously reported (Noam et al., 2010; Han et al., 2011). Membrane capacitance ($C_m$) was read directly from the compensation dial on the amplifier, and the mean capacitance value (14.4±1.0 pF, n=51) did not differ between conditions (p=0.36, Student's t-test). Current density values were calculated by dividing current amplitude by $C_m$. $I_h$ activation kinetics were best fit to a mono-exponential function (Han et al., 2011), and only traces with goodness-of-fit >0.985 were included in the final analysis. The voltage-dependent activation profile of $I_h$ was derived by fitting a Boltzmann equation to $I_h$ conductance values of individual traces at different potentials, as recently described (Han et al., 2011).

All data points represent mean ± standard error of the mean. Statistical significance was determined using the non-parametric Mann-Whitney test, Student’s t-test, or two-way ANOVA as indicated, with a p-value of p<0.05 to indicate significant difference.
Chapter 5

Results

Deletion of the FLNa-interacting domain in HCN1 channels leads to augmented $I_h$ and altered gating properties

Depletion of FLNa was previously shown to augment the amplitude of HCN1-mediated $I_h$ and to facilitate its gating in HCN1-expressing melanoma cell lines (Gravante et al., 2004). To investigate the molecular basis of FLNa-mediated regulation of $I_h$, we generated a GFP-fused HCN1 channel mutant (HCN1[D22]GFP) lacking the 22 amino acids that form the FLNa-interacting domain (Table 1). In line with previous reports (Noam et al., 2010), the mere fusion of GFP to either the C'- or the N'-terminus of the channel did not influence its biophysical characteristics (Sup. Fig 1A-C). Step-wise membrane hyperpolarization of HCN1[D22]GFP-expressing HEK293 cells yielded slowly-activating, non-inactivating inward currents typical of $I_h$; these were virtually absent in mock-transfected cells (Sup. Fig 1D,F). These results indicate that removal of the FLNa-interacting domain from HCN1 is not detrimental to channel function or to its delivery to the cell membrane.

Having established a functional HCN1 channel lacking the FLNa-binding domain, we next studied the functional implications of FLNa-HCN1 interaction by recording $I_h$ from HEK293 cells that co-express DsRed-fused FLNa (FLNa_DsRed) with either HCN1_GFP or HCN1[D22]GFP. $I_h$ density was increased two-fold in HCN1[D22]GFP + FLNa_DsRed expressing cells compared with cells expressing HCN1_GFP + FLNa_DsRed (74.7±16.4 vs 149.4±27.8 pA/ pF; n=25,26 cells; p<0.05, Mann-Whitney test; Fig. 1), in accord with a previous study reporting $I_h$ up-regulation in FLNa-deficient melanoma cells (Gravante et al., 2004).

In the presence of over-expressed FLNa_DsRed, the gating properties of HCN1[D22]GFP were altered when compared to HCN1_GFP with a depolarizing shift of the voltage-activation profile ($V_{50} = -76.9±1.2$ vs -72.0±1.8 mV for HCN1_GFP, HCN1[D22]GFP respectively; n=7,6 cells; p<0.05; Fig. 1) and with no difference in the slope factor (k=7.4±0.7, 7.3±0.7; n.s.). In line with facilitated gating, the activation kinetics of HCN1[D22]GFP were accelerated compared to HCN1_GFP (two-way ANOVA; p<0.05). Finally, no differences were found between the current-voltage relationship of the two constructs or their reversal potential values ($E_{rev} = -42.1±2.0$ vs -39.3±0.8 mV for HCN1_GFP, HCN1[D22]GFP respectively; n=3,4 cells; n.s.). These latter data suggest that the FLNa-interacting domain of the HCN1 channel does not play a role in ion selectivity.
Regulation of HCN channels by filamin A

Figure 1 - Deletion of the FLNa-binding domain in HCN1 alters $I_h$ density and gating. A, Whole-cell responses to voltage hyperpolarizing steps in transfected HEK293 cells. $I_h$ was not detected in mock-transfected neurons where only DsRed was expressed (left). Co-expression of FLNa/DsRed with either HCN1\_GFP or HCN1[Δ22]\_GFP yielded inward currents with typical $I_h$-like properties, possessing larger current amplitudes in HCN1[Δ22]\_GFP-expressing cells. B, Quantitative summary revealed a two-fold increase in $I_h$ density in cells expressing HCN1[Δ22]\_GFP compared with HCN1\_GFP-expressing cells (numbers on bars indicate n of cells per experimental group). C, Example traces comparing the kinetics of HCN1\_GFP (black) and HCN1[Δ22]\_GFP (grey) upon stepping to different membrane potentials (from -40 mV), as indicated. The amplitude of the currents was scaled to allow for visual comparison of the time course. Only a portion of the initial response is shown. D, activation kinetics of $I_h$ in HCN1\_GFP- and HCN1[Δ22]\_GFP-expressing cells. HCN1[Δ22]\_GFP-mediated $I_h$ was faster than HCN1\_GFP (two-way ANOVA, p<0.05). E, Voltage-dependent activation of $I_h$ in HCN1\_GFP- and HCN1[Δ22]\_GFP-expressing
HEK293 cells. A depolarizing shift in the activation profile of HCN1 [Δ22]_GFP was observed, quantified by the half-activation ($V_{50}$) value (inset) of individual Boltzmann fits (see Chapter 3 for further details). 

F. Example $I_h$ traces representing the protocol used to determine the reversal potential ($E_{\text{rev}}$) of the current. Upon initial step to a near-maximal activation voltage of -110 mV, membrane potential was stepped back to a range of voltage values. Because $I_h$ de-activates relatively slowly, the instantaneous peak values of the tail current (inset on the right) represent predominantly the changes in driving force.

G. Current-voltage (I-V) curves, plotted based on the protocol shown in F. No difference was found between $E_{\text{rev}}$ of $I_h$ in HCN1[GFP]_ and HCN1[Δ22]_GFP -expressing cells (note: standard error in many data points was too small to be visualized). All data points represent mean ± s.e.m. *=p<0.05, Student’s t-test. In all experiments, FLNaDsRed was expressed alongside HCN1 channel constructs at a 1:1 cDNA ratio.

Channel isoform-specific clustering of HCN channels by FLNa

The results described above suggest an inhibitory role for FLNa on $I_h$ amplitude and gating properties. Inhibition of $I_h$ could derive from a direct change in the properties of the membrane-inserted channel, or could arise from the presence of fewer molecules on the cell surface. Therefore, we next tested whether FLNa influenced HCN channel trafficking, using confocal imaging of HEK293 cells transfected with DNA constructs designed to selectively target HCN1-FLNa interaction (Table 1). Remarkably, co-expression of HCN1[GFP] channels with FLNaDsRed resulted in strong clustering of HCN1 channels, apparent as large, distinct GFP-positive puncta (Fig. 2A), which were absent when FLNaDsRed was co-expressed with either HCN1[Δ22]_GFP or HCN2[GFP] (Fig. 2B,C). Note that these effects were easily observable in co-transfected cells, where both HCN1[GFP] and HCN1[Δ22]_GFP were over-expressed. Although HEK293 cells do express FLNa (Sup. Fig. 2), the low endogenous levels, compared with the over-expression of transfected HCN channels were not optimal for demonstrating the effects of HCN-FLNa interaction.

To rule out potential artifacts mediated by the fusion of the GFP molecule to the HCN1 channel, we employed a second GFP-fused HCN1 channel construct where the GFP was fused to the N’, rather than to the C’ terminus (HCN1[GFP,N’]). Co-expressing HCN1[GFP,N’] with FLNaDsRed recapitulated the results obtained with HCN1[GFP] (Sup. Fig 3), confirming that the observed clustering of HCN1 was unrelated to the fluorophore fusion.

The FLNa-dependent effects on HCN1 distribution pattern may be mediated either by conformational changes due to binding of FLNa to the channel, or by crosslinking HCN1 to cytoskeletal elements. To discern between these two possibilities, we employed a DsRed-fused, FLNa truncation mutant (FLNa[23-24]_DsRed), which possesses the HCN1-binding region (namely, the C’ terminal IgG like domains 23-24, see Experimental Procedures), but
does not possess the actin binding regions. In accord with the lack of actin-binding capacity, the distribution of FLNa[23-24]DsRed in HEK293 cells was diffuse and non-specific, as opposed to the filamentous distribution observed with FLNaDsRed and endogenous FLNa (Fig. 2D, Sup. Fig 2). Co-expression of HCN1\textsubscript{GFP} with FLNa[23-24]DsRed resulted in a relatively diffuse distribution that resembled the expression pattern of HCN1[Δ22]\textsubscript{GFP} when expressed with FLNaDsRed (Fig. 2D), indicating that FLNa-induced clustering of HCN1 channels requires a full-length, actin binding FLNa protein. Co-expression of FLN[23-24]DsRed with either HCN1[Δ22]\textsubscript{GFP} or HCN2\textsubscript{GFP} resulted in a smooth distribution pattern (not shown), as observed upon expression of these HCN channel constructs with the full-length FLNaDsRed. These results, showing the need for the actin-interacting domain of FLNa to enable HCN1 clustering, support a mechanism involving a molecular complex consisting of HCN1, FLNa and actin. Such a complex might influence HCN1 channel trafficking either by regulating channel distribution on the cell surface or by association with intracellular organelles involved in channel transport and turnover.
Figure 2 - Filamin-dependent clustering of HCN1 channels. Confocal images of HEK293 cells expressing HCN1\(_{\text{GFP}}\) (A), HCN1[Δ22]\(_{\text{GFP}}\) (B), or HCN2\(_{\text{GFP}}\) (C) alongside with FLNa\(_{\text{DsRed}}\). Whereas expression of FLNa\(_{\text{DsRed}}\) with HCN1\(_{\text{GFP}}\) lead to a clustered appearance of HCN1\(_{\text{GFP}}\) (A), the distribution pattern of both HCN1[Δ22]\(_{\text{GFP}}\) (B) and HCN2\(_{\text{GFP}}\) (C) was diffuse. D, A confocal image of a HEK293 cell, displaying a diffuse distribution pattern when HCN1\(_{\text{GFP}}\) is expressed alongside a truncated filamin mutant (FLNa[23-24]\(_{\text{DsRed}}\)) that includes only the HCN1-binding region but not the actin-binding
portion of the protein. All images are of a single optical slice in the z plane (<1 μm). Scale bars = 10 μm.

**FLNa-mediated accumulation of HCN1 channels in lysosomal compartments**

Because FLNa is involved in the endocytic processing of several membrane proteins (Liu et al., 1997; Cho et al., 2007; Sverdlov et al., 2009; Minsaas et al., 2010), we asked whether the FLNa-dependent accumulation of HCN1 channels in intracellular clusters may represent altered endocytic localization. Immunocytochemistry of HEK293 cells expressing both HCN1<sub>GFP</sub> and FLNa<sub>DsRed</sub> using an antibody directed against the lysosomal protein LAMP1, revealed accumulation of HCN1 in lysosomal compartments (Fig. 3A,D): HCN1 clusters co-localized with LAMP1-positive organelles. Some of the HCN1 clusters were also localized to early endosomes (albeit to a lesser degree than lysosomes), as evident by confocal imaging of cells immunolabeled with the early endosomal marker EEA1 (Fig. 3B,D). To examine the presence of HCN1 in recycling endosomes, a transferrin-uptake assay was carried out, using an alexa-633-conjugated transferrin on HCN1<sub>GFP</sub>+FLNa<sub>DsRed</sub>–expressing HEK293 cells. In contrast to their robust presence in lysosomes, only a small sub-population of HCN1 clusters was localized to recycling endosomes (Fig. 3C,D). Taken together, these data suggest that FLNa influences the subcellular localization (and likely the trafficking) of HCN1 channels into organelles involved in the internalization and degradation of membrane proteins.
Figure 3 - HCN1 clusters represent sub-populations of endosomal organelles. Confocal images of HEK293 cells transfected with HCN1$_{GFP}$ and FLNa$_{DsRed}$ and immuno-labeled with an antibody directed against the lysosomal marker LAMP1 ($A$) or against the early endosomal marker EEA1 ($B$). Visualization of recycling endosomes was carried out using the Tf-uptake assay with an alexa-633 – conjugated transferrin ($C$). $D-F$, Magnified regions from panels $A-C$ (as indicated), demonstrating the strong colocalization of HCN1$_{GFP}$ clusters with LAMP1-positive organelles and the modest presence of HCN1$_{GFP}$ in early endosomes and recycling endosomes. Images in $A-C$ are a stack of optical slices in the z-plane, whereas the magnified regions in $D-F$ are single z-slices (optical thickness<$1$ $\mu$m). Scale bars = $10$ $\mu$m.
FLNa-induced HCN1 clustering and $I_h$ inhibition are dynamin-dependent and reversible

The accumulation of HCN1 channel protein in endosomes implies a role for FLNa in the removal of HCN1 channels from the cell membrane. The GTPase dynamin contributes crucially to many forms of endocytosis by enabling the fission of newly formed pits budding from the plasma membrane (Macia et al., 2006). A 4-hour incubation of FLNa$_{DslRed}$ + HCN1$_{GFP}$-transfected HEK293 cells with the selective dynamin-inhibitor dynasore led to a marked decrease in HCN1 channel clustering (Fig. 4A), confirming a role for dynamin-dependent internalization in HCN1 clustering. Importantly, dynasore treatment not only rescued the clustered phenotype, but also augmented $I_h$ density two-fold in HCN1$_{GFP}$ + FLNa$_{DslRed}$ -transfected HEK293 cells (54±10 vs 106±19 pA/pF for vehicle and dynasore treatments, respectively; n=23,30 cells; p<0.05, Mann-Whitney test). The density of $I_h$ in the dynamin treated cells was comparable to that of HCN1[Δ22]$_{GFP}$ + FLNa$_{DslRed}$ -expressing cells (Fig. 4B). Incubation of HCN1[Δ22]$_{GFP}$ + FLNa$_{DslRed}$ -transfected cells with dynasore did not increase $I_h$ density (104±16 vs 100±21 pA/pF for vehicle and dynasore treatments, respectively; n=20,20; Mann-Whitney test). Notably, in cells expressing FLNa with the HCN1-channel mutant that lacks the FLNa binding domain, dynasore did not influence channel distribution (not shown). These findings suggest that the lack of FLNa interaction confers higher stability of HCN1 channels within the membrane and a slower internalization rate.
Figure 4 – FLNa-dependent HCN1 clustering and $I_h$ inhibition are dynamin-dependent and reversible. A, Confocal images of HEK293 cells that co-express HCN1$_{GFP}$ and FLNa$_{DsRed}$, showing reduced HCN1 clustering following incubation with the selective dynamin-inhibitor dynasore. The images are a stacked representation of optical slices (z) with $<$1 μm thickness per slice. The green channel represents HCN1$_{GFP}$ signal, whereas insets in red represent the signal emitted from FLNa$_{DsRed}$ in the same cell. B, Example traces of $I_h$, recorded from HCN1$_{GFP}$ +FLNa$_{DsRed}$ –expressing HEK293 cells pre-treated with either vehicle (0.26% DMSO) or dynasore. C, Quantitative summary of $I_h$ density in cells expressing either HCN1$_{GFP}$ +FLNa$_{DsRed}$ or HCN1[Δ22]$_{GFP}$ + FLNa$_{DsRed}$* pre-treated with either vehicle or dynasore. Exposure to dynasore resulted in two-fold increase of $I_h$ density in HCN1$_{GFP}$ +FLNa$_{DsRed}$ –expressing cells, whereas $I_h$ density in HCN1[Δ22]$_{GFP}$ + FLNa$_{DsRed}$ expressing cells was insensitive to dynasore.
FLNa-induced alterations of HCN1 channel surface expression

The data presented so far suggest that FLNa promotes the elimination of HCN1 channels from the cell membrane. To directly test whether FLNa influences HCN1 surface expression, we employed total internal reflection fluorescent microscopy (TIRF-M), a technique that allows the visualization of only a very thin section of the cell (50-150 nm), representing membrane and peri-membranous domains (Jaiswal and Simon, 2007). To discern the signal of surface-expressed channels from intracellular ones we obtained both widefield images (at a right angle) and TIRF-M images of the same cells. A comparison of widefield and TIRF-M images revealed a sub-population of HCN1\textsubscript{GFP} clusters localized to peri-membranous regions, whereas other clusters were localized to intracellular compartment (Fig. 5A,E-F). In contrast to the punctate surface expression of HCN1\textsubscript{GFP} in the presence of FLNa, the surface distribution pattern of both HCN2\textsubscript{GFP} and HCN1[Δ22]\textsubscript{GFP} was diffuse (Fig 5B,C). This was also the case for HCN1\textsubscript{GFP} in the presence of FLNa[23-24]\textsubscript{DsRed}, the truncated FLNa construct that lacks the actin binding domains (Fig. 5D). Taken together, these results imply that interaction of HCN1 with FLNa results both in HCN1 channel clustering on the cell surface, as well as in intracellular accumulation of channel protein via endocytosis.
Figure 5 - Intracellular and surface localization of HCN1 clusters revealed by TIRF-M imaging. 

A-D, Widefield and TIRF images of cells expressing FLNa and HCN channel constructs. A, Widefield images of both FLNa_DsRed (evident by their DsRed signal) and HCN1_GFP (GFP signal) are shown, as well as TIRF images of the GFP signal obtained from the same cells. The right column (#4) represents a magnified region of the TIRF image to its left. Note the clustered appearance of HCN1_GFP when expressed alongside FLNa_DsRed under TIRF conditions. B-C, Widefield and TIRF images of HCN1[D22]GFP (B) and HCN2_GFP (C), expressed in the presence of FLNa_DsRed. D, Widefield and TIRF images of HCN1_GFP co-expressed with FLNa[23-24]DsRed. E-F, A close examination of widefield and TIRF images reveals that some of the HCN1_GFP clusters observed in the presence of FLNa_DsRed are near the cell surface (as evident by their presence in both widefield and TIRF images, green arrows), whereas others are intracellular (as evident by their virtual disappearance from the TIRF image, light blue arrows). Scale bars = 5 μm.
Discussion

To investigate the roles of FLNa in regulating HCN1 channel trafficking and function, we combined a mutational approach with confocal imaging, TIRF microscopy and electrophysiology to investigate the roles of FLNa in regulating HCN1 channel trafficking and function. The key findings of this study are: (1) FLNa induces the clustering of HCN1 (but not of HCN2) channels in both peri-membranous and intracellular domains; (2) Intracellular clustering of HCN1 by FLNa represents accumulation of the channels in lysosomes, and is accompanied by reduced $I_h$ amplitude and altered gating; (3) Both FLNa-dependent HCN1 internalization and $I_h$ inhibition can be rescued by selective interference with the GTPase activity of dynamin.

Previous work in melanoma cell lines demonstrated increased $I_h$ amplitude, shifted voltage-gating and accelerated kinetics in cells devoid of FLNa (Gravante et al., 2004). Whereas the complete absence of FLNa influences many cellular properties such as motility, elasticity and endosomal localization (Liu et al., 1997; Stossel et al., 2001), our data support a selective role for FLNa in regulation of HCN1 channels: deletion of the FLNa binding domain in HCN1 channels up-regulated $I_h$ density and facilitated gating properties. A central finding of this study is the FLNa-dependent accumulation of HCN1 channels in lysosomes. Increased internalization and degradation of HCN1 channels is expected to result in net decrease of HCN1 channel abundance on the membrane, consistent with reduced $I_h$ density.

An intriguing aspect of HCN channel regulation by FLNa is its isoform-specificity, manifested by exclusive regulation of the HCN1 channel isoform by FLNa (Gravante et al. 2004). Isoform-specific regulation of HCN channels may alter $I_h$ properties (Brewster et al., 2002) and influence channel heteromerization (Brewster et al., 2005). Recently, we found subunit-specific distribution and trafficking dynamics of HCN channels in hippocampal neurons: whereas the expression of HCN1$_{GFP}$ channels was punctate and accompanied by dynamic vesicular transport along dendrites, HCN2$_{GFP}$ channels were characterized by a diffuse distribution pattern and reduced mobility kinetics (Noam et al., 2010). Interestingly, the trafficking dynamics of the HCN1 isoform in hippocampal neurons were strongly inhibited upon actin-stabilization, implying the tight involvement of the actin network in regulating HCN1 channel trafficking.

Little is known on the dynamics of HCN channel turnover within the membrane. Following internalization, membrane proteins can be sent to lysosomal degradation or alternatively be redirected to recycling (Maxfield and McGraw, 2004). HCN2 and HCN4 channels are readily observable in recycling endosomes, providing a pool of “spare” channels to be re-inserted
into the cell membrane upon stimulation (Hardel et al., 2008). Surprisingly, we found only a minority of HCN1 channels in this “recycling pool”. FLNa does not bind HCN2 or HCN4, and our results suggest that FLNa binding to HCN1 aggregates the channels on the cell surface, destining them to internalization and lysosomal degradation. The exact mechanism is not fully understood, but seems to involve specific interaction domains within the FLNa and HCN1 molecules as well as dynamin-mediated internalization. In general, FLNa can influence channel trafficking to lysosomal organelles by either controlling the location and stability of the channels within the membrane, or by regulating intracellular processes of protein sorting into distinct endocytic compartments. Considering the fact that we could not detect substantial presence of FLNa in HCN1-containing intracellular clusters or in other endocytic organelles (Fig. 3), it seems more likely that the regulation of HCN1 channels by FLNa occurs at--or in proximity--to the cell membrane.

Thus, based on the current evidence, we propose two alternative models for the action of FLNa on HCN1 channels: in the first model (Fig. 6A), a protein complex between HCN1, FLNa, actin, and other potential (yet to be identified) cytoskeletal proteins targets the channels to specialized microdomains on the membrane. This results in a non-homogenous distribution of HCN1 channel protein on the cell surface. In this scenario, the clustered localization of HCN1 channels on the membrane allows for a more efficient endocytosis by enabling the internalization of large channel aggregates in a single endocytic event. Increased efficiency of HCN1 channel internalization leads eventually to the accumulation of channel protein in lysosomes, resulting in net decrease of both surface and total channel protein, and decreased $I_h$ amplitude. An alternative model suggests that HCN1-FLNa interaction occurs throughout most of the cell surface, and rather than re-directing channels to specific microdomains (as proposed in model #1), FLNa promotes internalization along vast portions of the cell membrane (Fig 6B). Facilitating internalization may occur via complexes that include actin, as well as other adaptor proteins and dynamin. The clustered appearance of surface HCN1 in the second model may represent distinct regions on the membrane that are more resilient to internalization, and therefore contain large amounts of non-internalized channel protein. Further studies into the insertion, internalization and lateral diffusion of surface HCN1 channels are required to fully distinguish between these two models.
Figure 6 – Models of HCN1 clustering and internalization by FLNa. The cartoons depict two alternative models explaining the clustering effect of FLNa on both surface and intracellular HCN1 channels, and their accumulation within lysosomes. According to model #1 (depicted in A), HCN1-FLNa interaction localizes HCN1 channels into distinct microdomains within the membrane, resulting in their clustering (top panel). These domains facilitate an efficient internalization of HCN1 by enabling the endocytosis of large volume of protein in a single internalization event. Whereas some endosomes may find their way back to the membrane through recycling, the continuous internalization would eventually result in net accumulation in lysosomes (bottom panel). An alternative model (B) suggests that interaction between FLNa and HCN1 leads to internalization of the channels across vast portions of the membrane (top panel), while the clusters observed on the surface represent sub-regions where internalization occurs at lower rate, therefore “rescuing” the channels from endosomal fate (lower panel). However, the continuous internalization from the majority parts of the membrane results in accumulation of HCN1 channels in lysosomes. C, In the absence of FLN-HCN1 interaction, a more diffuse membrane distribution is observed either due to lack of specific targeting within the membrane (according to model #1) or due to reduced internalization (according to model #2). Because of the enhanced stability of the channels within the membrane, less channel molecules are present in organelles of the endocytic pathway.
The involvement of FLNa in membrane protein trafficking is not unique to HCN channels. Several ion channels and receptors have been reported to interact with FLNa, including dopamine D2/D3 receptors (Lin et al., 2001; Li et al., 2000), Kir 2.1 channels (Sampson et al., 2003), mu-opioid receptors (Onoprishvili et al., 2003), and the Kv4.2 potassium channels (Petrecca et al., 2000). Whereas in some cases FLNa increases the stability of proteins within the cell membrane (Petrecca et al., 2000; Sampson et al., 2003; Seck et al., 2003; Beekman et al., 2008), in other cases it may promote internalization and sequestration (Cho et al., 2007; Minsaas et al., 2010). Thus, the effect that FLNa exerts on its binding partner may be influenced by many factors, including the identity of the interacting protein, and may further result in the co-regulation of different membrane proteins.

Regulation of HCN channels by auxiliary proteins is emerging as a key mechanism by which the trafficking and biophysical properties of HCN channels can be modulated. The brain-specific auxiliary protein TRIP8b is a potent regulator of HCN1 and HCN2 channels, influencing both their surface expression and gating properties (Lewis et al., 2009; Santoro et al., 2009; Zolles et al., 2009; Han et al., 2011). Additional HCN-interacting proteins have also been reported (Yu et al., 2001; Kimura et al., 2004). Interestingly, TRIP8b has been recently found to influence HCN channel surface expression via control of internalization and lysosomal targeting: TRIP8b splice variants that promote surface expression inhibit channel internalization (Lewis et al., 2011), and TRIP8b variants that reduce HCN surface expression increase lysosomal targeting (Popova et al., 2011). Our findings on the roles of the actin-binding protein FLNa in promoting HCN1 channel internalization and degradation suggest that multiple mechanisms and auxiliary proteins may participate in regulation of HCN channel turnover. Studies into the combinatorial effects of these mechanisms in different physiological contexts will promote our understanding of the dynamic control of channel surface expression, and their implications.

In summary, the results presented in this study imply an inhibitory role for the actin binding protein FLNa on the membrane expression of HCN1 channels, mediated by increased internalization and lysosomal targeting. Our results suggest isoform-specific mechanisms that control the endocytic targeting and stability of HCN channels within the cell membrane, processes that may underlie dynamic regulation of the channels in a variety of physiological contexts.
Acknowledgments

We Thank Dr Bina Santoro for the N’ terminal GFP fused HCN1 and HCN2 constructs, Dr Dane Chetkovich for the HCN1_{GFP} construct, and Dr Fumi Nakamura for the DsRed-fused filamin A construct. We also thank Eng. Ronald Breedijk from the Center for Advanced Microscopy at the University of Amsterdam for expert assistance with TIRF imaging.
Supplemental Figure 1 – Functional expression of HCN channels in HEK293 cells. Example traces of whole-cell responses to hyperpolarizing steps (from -40 to -130 mV, in 10 mV decrements) of transfected HEK293 cells. A, Expression of untagged mouse HCN1 (alongside with EGFP, at 1:1 ratio), resulted in a typical I_h with HCN1-like properties. B-C, both C’- and N’ terminus fusion of GFP to HCN1 yielded I_h with comparable properties. D, Deletion of the FLNa-binding domain in HCN1 was not detrimental to channel function, as evident by the typical I_h traces. E, Expression of GFP-fused HCN2 channels resulted in I_h with slower kinetics and altered gating, as expected for this channel isoform (Biel et al., 2009). F, Endogenous I_h could not be detected in HEK293 cells, as shown in a cell transfected with only DsRed.
Supplemental Figure 2 – Distribution patterns of endogenous and exogenous FLNa in HEK293 cells. Left: The distribution pattern of native FLNa in HEK293, as demonstrated by immuno-labeling of a non-transfected HEK293 cells with a monoclonal antibody directed against FLNa. Note the typical filamentous distribution of FLNa in filopodia tips. Center: A distribution pattern similar to that of native FLNa is observed in DsRed-fused FLNa (FLNa_{DsRed})-expressing HEK293 cells. The image represents the DsRed signal emitted solely from the transfected FLNa_{DsRed}. Right: In contrast to native FLNa and exogenous FLNa_{DsRed}, expression of a truncated mutant of FLNa that includes only the last two IgG-like domains (which form the HCN1 binding domain) but not the actin-binding domains results in non-specific, homogenous distribution of the protein. All images are a z-stack representation of confocal optical slices at <1 μm thickness. Scale bars = 10 μm.

Supplemental Figure 3 - FLNa-dependent clustering of N’ terminus-fused GFP-HCN1 channel protein. Similar to the results obtained with HCN1_{GFP-N’}, co-expression of HCN1_{GFP,N’} with FLNa_{DsRed} resulted in intense clustering (top panel), whereas co-expression of HCN1_{GFP,N’} with truncated FLNa (FLNa[23-24]_{DsRed}) resulted in a more continuous distribution pattern (bottom panel). Scale bars = 10 μm.