

was to establish a model to characterize  $\alpha_{1D}$  Ca current using the novel technology of RNA interference.

**Methods and Results:** Rat neonatal cardiomyocytes (RNC) were transfected with  $\alpha_{1C}$  specific siRNA using lipofectamine which resulted in 50% silencing at the mRNA/protein level. Limited by the low transfection efficiency (50%) in the RNC, we cotransfected  $\alpha_{1C}$  siRNA with cy3 labeled Human GAPDH siRNA and sorted out the fluorescent cells using FACS to separate transfected and non-transfected cells. The level of silencing of  $\alpha_{1C}$  in enriched transfected cells reached only 65% efficiency. To achieve higher transfection efficiency, we generated and infected RNC with a lentivirus construct carrying the  $\alpha_{1C}$  siRNA sequence under a U6 promoter. Using this model, we achieved 100% transfection efficiency, and more than 90% silencing of the  $\alpha_{1C}$  gene confirmed by real-time PCR, Western blot, and immunofluorescence. These biochemical results were confirmed electrophysiologically by measurements of total L-type Ca current which was reduced by 80% in transfected cells.

**Conclusion:** Lentiviral shRNA is an efficient model for post-transcriptional gene silencing of ion channels in primary cardiomyocytes. This novel approach provides a valuable mean for assessing the differential roles of  $\alpha_{1C}$  and  $\alpha_{1D}$  Ca channels in native cardiomyocytes and could be used to examine their roles in physiological and pathological settings.

### 935-Pos Board B814

#### Construction Of Functional N-type $\text{Ca}^{2+}$ Channels ( $\text{Cav}2.2$ ) With Accessible External Epitope Tags Suitable For Live Cell Labeling

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Recent studies of voltage-gated  $\text{Ca}^{2+}$  channels indicate that mechanisms other than alterations in gating contribute to modulation. For example, internalization of receptor/channel complexes (e.g., ORL1) or formation of non-conducting species (e.g., RGK proteins), potentially contribute to  $\text{Ca}_v2.2$  function. An essential technique for investigating these phenomena is the ability to specifically label fully functional  $\text{Ca}_v2.2$   $\alpha$ -subunits (*CACNA1B*) in the plasma membrane of living cells. Towards this end, we attempted a systematic insertion of a hemagglutinin (HA) epitope tag into each of the 12 predicted extracellular loops of the rat  $\text{Ca}_v2.2$   $\alpha$ -subunit. The first round of mutagenesis resulted in the addition of sites to 1E1 (domain 1, extracellular loop 1), 1E2, 2E2, 3E1, 3E2 and 4E3. A sequence coding for a short flexible linker plus two contiguous copies of the hemagglutinin epitope was ligated into each site. The resulting constructs were electroporated into HEK293 cells along with plasmids encoding  $\text{Ca}^{2+}$  channel  $\beta_{2a}$  and  $\alpha_{2\delta}$  subunits, and the  $\text{K}^+$  channel IRK1. Robust "rim type" immunofluorescent labeling (in living cells) was detected for 4 of 6 clones (1E2, 3E1, 3E2 and 4E3). Expression of these constructs in HEK293 cells produced channels with electrophysiological properties similar to wildtype as determined by whole-cell patch-clamp with 10 mM  $\text{Ca}^{2+}$  as the charge carrier. Average peak currents were (in nA)  $-5.4 \pm 0.7$  for the control and  $-3.8 \pm 0.7$ ,  $-1.2 \pm 0.2$ ,  $-3.1 \pm 0.7$  and  $-4.0 \pm 0.7$  for 1E2, 3E1, 3E2 and 4E3, respectively. The IV curves and individual current trajectories for each clone were superficially similar to the wildtype. These clones should provide powerful tools for the study of trafficking and modulatory mechanisms of  $\text{Ca}_v2.2$ . Additionally, the tagging strategy may be applicable to additional members of the  $\text{Ca}_v$  and  $\text{Na}_v$  families of voltage-gated channels.

### 936-Pos Board B815

#### Role Of Gamma Subunit In The Targeting Of Functional Cardiac L-Type $\text{Ca}^{2+}$ Channels

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The cardiac isoform  $\gamma 6$  of the  $\text{Ca}^{2+}$  channel  $\gamma$  subunit family has been proposed to inhibit T-type  $\text{Ca}^{2+}$  channels by reducing the channel's availability for activation (Hansen et al., 2004, J Mol Cell Cardiol). Here we investigated how the  $\gamma 6$  subunit (long transcript) regulates L-type  $\text{Ca}^{2+}$  channels expressed in tsA201 cells.

First, the sub-cellular localization of the N-terminally GFP-tagged  $\gamma 6$  was visualized by confocal imaging. Expressed alone or with any combination of other types of subunits, the  $\gamma 6$  targeted well to the plasma membrane. Second, when the  $\gamma 6$  subunit (long transcript) was co-expressed with  $\alpha_{1C}$  and  $\beta_{2a}$  subunits, it dramatically reduced the density of ionic currents recorded with 10 mM  $\text{Ba}^{2+}$  from  $142 \pm 26$  pA/pF ( $n=4$ ) in control to  $3 \pm 2$  pA/pF ( $n=7$ ). The gating charge was also nearly eliminated in the presence of the  $\gamma 6$  subunit (from  $47 \pm 12$  fC/pF in control to less than 5 fC/pF in cells with the  $\gamma 6$ ). The reduction in current and gating charge was independent on GFP-tagging of the  $\gamma 6$ .

Taken together, these results indicate that the cardiac  $\gamma 6$  subunit regulates expression/functional targeting of cardiac L-type  $\text{Ca}^{2+}$  channels.

### 937-Pos Board B816

#### Calreticulin Negatively Regulates the Surface Expression of $\alpha_{1D}$ L-Type Calcium Channel

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**Background:** Quality control of several proteins is strictly regulated by molecular chaperones in the endoplasmic reticulum (ER). Calreticulin, an ER  $\text{Ca}^{2+}$ -binding chaperone, has been shown to regulate the surface expression of several membrane proteins including the cystic fibrosis transmembrane conductance regulator (CFTR) which under oxidative stress results in its internalization and proteasomal degradation. Decrease of L-type Ca current and channel protein has been described in autoimmune associated congenital heart block. Here, we demonstrated a novel mechanism of down-regulation of  $\alpha_{1D}$  L-type Ca channel using native human fetal cardiac cells and tsA201 cell line.

**Methods and Results:** Using Confocal microscopy, we found surface staining of calreticulin on cultured human fetal cardiomyocytes (HFC) gestational age 18-24 weeks. Coimmunoprecipitation from HFC using anti- $\alpha_{1D}$  antibody, and probing with anti-calreticulin antibody revealed a 46 kDa band corresponding to calreticulin. Overexpressing calreticulin in human embryonic kidney cells (tsA201) resulted in a decrease in surface expression of  $\alpha_{1D}$  L-type Ca Channel. Electrophysiological studies showed that co-transfection of calreticulin with  $\alpha_{1D}$  L-type Ca Channel led to 55% inhibition of the  $\alpha_{1D}$  Ca current expressed in tsA201 cells.

**Conclusions:** These results show the first evidence that calreticulin: 1) is found on the cell surface of human fetal cardiomyocytes; 2) is coimmunoprecipitated with  $\alpha_{1D}$  L-type Ca Channel; 3) negatively regulates  $\alpha_{1D}$  surface expression; 4) decreases  $\alpha_{1D}$  Ca current in tsA201 cells co-expressed with  $\alpha_{1D}$  and calreticulin. The data demonstrated a novel mechanism of modulation of  $\alpha_{1D}$  Ca channel, which may be involved in numerous pathological settings such as congenital heart block.

### 938-Pos Board B817

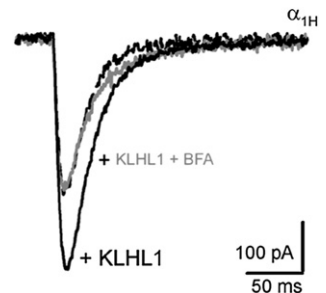
#### Inhibition Of Recycling Endosomes By Brefeldin-A Prevents KLHL1-mediated Upregulation Of $\alpha_{1H}$ T-type Currents

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The Kelch-like 1 (KLHL1) actin binding protein interacts with  $\alpha_{1H}$  calcium channels and increases calcium current density *via* interaction with the actin cytoskeleton, resulting in an increase in the number of channels at the membrane. We probed the effect of Brefeldin A (BFA), which affects protein transport *via* disruption of the trans-Golgi network and by blocking vesicle recycling. HEK 293 cells stably transfected with  $\alpha_{1H}$  were incubated with various BFA concentrations; overnight incubations resulted in the elimination of basal  $\alpha_{1H}$  currents ( $\text{IC}_{50}=21 \mu\text{M}$ ), consistent with impaired channel trafficking from the Golgi complex. Interestingly, low [BFA] (107 nM) and/or short-term incubations (3.6  $\mu\text{M}$  for 1 hr) did not alter endogenous  $\alpha_{1H}$  levels, yet completely eliminated the KLHL1 effect. Current deactivation kinetics changes originally observed in the presence of KLHL1 persisted in the presence of BFA, indicating that BFA does not affect the direct interaction of KLHL1 with  $\alpha_{1H}$  already present at the plasma membrane.

Our data suggests KLHL1 up-regulates channel number by increasing  $\alpha_{1H}$  re-insertion into the membrane *via* recycling endosomes, a process that involves stabilization of the actin cytoskeleton. Supported by AHA-0615508Z (KA) and NSF-0641141 (EPR).



### 939-Pos Board B818

#### Cardiac L-type Ca Channel as an Oxygen Sensor; Possible Involvement of Ca/Calmodulin Binding Domain

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Cellular oxygen sensing is defined as the ability of a cell to respond to changes in extracellular oxygen pressure through regulation of membrane ionic currents, mitochondrial oxidative phosphorylation or transcription of regulatory proteins. In the heart hypoxia has been shown to alter ionic currents through mitochondrial redox regulation and phosphorylation by kinases. Here we report