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The MAP1B-LC1/UBE2L3 complex catalyzes degradation of cell surface $Ca_V2.2$ channels

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We reported recently a new mechanism by which the neuronal N-type Ca^{2+} ($Ca_V2.2$) channel expression may be regulated by ubiquitination. This mechanism involves the interaction between the channel and the light chain (LC1) of the microtubule associated protein B (MAP1B). We also showed that MAP1B-LC1 could interact with the ubiquitin-conjugating E2 enzyme UBE2L3 and that the ubiquitination/degradation mechanism triggered by MAP1B-LC1 could be prevented by inhibiting the ubiquitin-proteasome proteolytic pathway. We now report that MAP1B-LC1 can interact with the 2 main variants of the $Ca_V2.2$ channels ($Ca_V2.2e37a$ and $Ca_V2.2e37b$) and that the MAP1B-LC1-mediated regulation most likely involves an internalization of the channels via a dynamin and clathrin-dependent pathway. In addition, here we propose that this novel mechanism of Ca_V channel regulation might be conserved among N-type and P/Q-type channels.

Keywords: Brefeldin A, Dynasore, LC1, MAP1B, N-type channels, trafficking, UBE2L3

Abbreviations: MAP1B-LC1, Microtubule-associated protein 1B light chain; UBE2L3, Ubiquitin-conjugating enzyme E2 L3; UPS, Ubiquitin proteasome system; HVA, high-voltage-activated; BFA, brefeldin A; DRG, dorsal root ganglion.

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Introduction

The voltage-gated Ca^{2+} channel family (Ca_V1-Ca_V3) plays important roles in the physiology of excitable cells. The Ca_V1 (L-type) and Ca_V2 (N-, P/Q- and R-type) channel subfamilies are heteromultimers formed by an ion-conducting α_1-subunit plus auxiliary $\alpha_2\delta$-, β- and γ-subunits.[1-3] The N-type ($Ca_V2.2$) channels are present in both the central and peripheral nervous systems, and though they act as major Ca^{2+} entry pathways to support neurotransmitter release at synapses,[1] the cellular and molecular mechanisms that control the functional expression of these channels are not well understood.

It is well-known that Ca_V2 channels interact with multiple binding partners that regulate gating properties and membrane localization. Likewise, recent studies have recognized ubiquitination/degradation as an important long-term mechanism by which channel expression can be regulated.[4] In this context, we have recently published evidence that the light chain 1 (LC1) of the microtubule associated protein B (MAP1B) may be involved in $Ca_V2.2$ channel functional expression.[5] This newly identified interaction involves a binding sequence in the N-terminal half of MAP1B-LC1 and binding domains within the C terminus of the $Ca_V2.2\alpha_1$ subunit. Furthermore, the $Ca_V2.2\alpha_1$/MAP1B-LC1 complex may interact with the E2 conjugase of the ubiquitin proteasome system UBE2L3, suggesting that MAP1B-LC1 may act as a scaffold protein to favor UBE2L3-mediated channel ubiquitination.

Here, we extend those results to show that the MAP1B-LC1-mediated regulation may involve an internalization of the $Ca_V2.2$ channels via a dynamin and clathrin-dependent pathway, and that the ubiquitination/degradation mechanism triggered by MAP1B-LC1 might be conserved among N-type and P/Q-type channels. Therefore, these data add further support to the idea that ubiquitination may play an important role in the regulation of Ca_V2 channel surface expression.

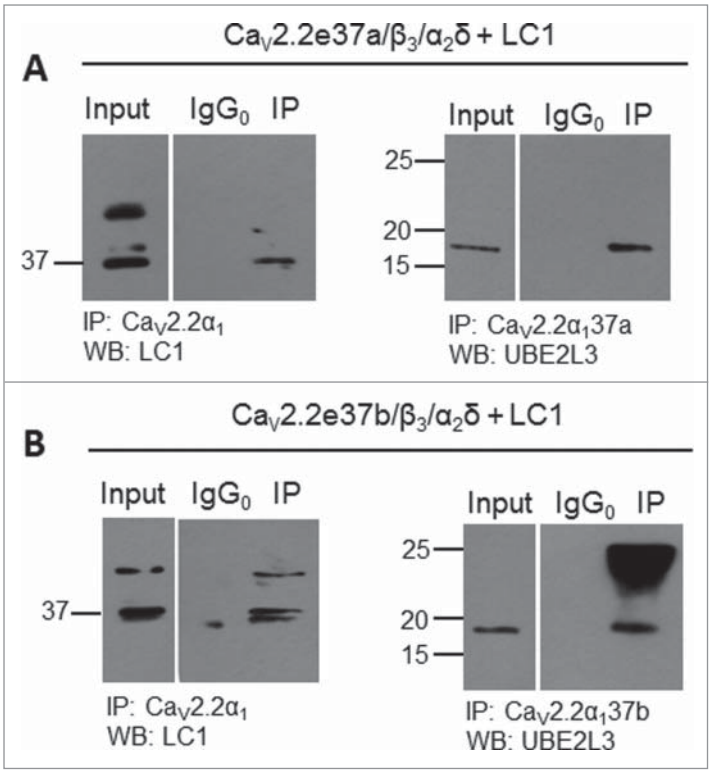

Figure 1. The protein complex UBE2L3/LC1 can interact with the 2 splice variants of the $Ca_V2.2\alpha_1$ subunit. Proteins from HEK-293 cells co-transfected with LC1-myc and the 2 e37 splice variants of the $Ca_V2.2\alpha_1$ pore-forming subunit $Ca_V2.2\alpha_1$e37a (**A**) and $Ca_V2.2\alpha_1$e37b (**B**) were immunoprecipitated with anti-$Ca_V2.2\alpha_1$, or control antibodies and the bound proteins were analyzed by Western blot using an antibody against MAP1B-LC1 and UBE2L3. Positive interactions were observed in both cases ($n = 2$). The irrelevant control (IgG_0) was a rabbit anti-Pet antibody.

Results and Discussion

As mentioned earlier, we recently revealed that the surface density of neuronal $Ca_V2.2$ channels is regulated through their interaction with the MAP1B-LC1 protein, and that the ubiquitin-proteasome pathway plays a key role in this process.[5] Likewise, previous studies have shown that alternative pre-mRNA splicing of a pair of ~30 amino acid encoding exons in the C-terminus of the $Ca_V2.2\alpha_1$ subunit, e37a and e37b, underlie the expression of 2 mutually exclusive N-type channel isoforms.[6-8] These channels show different expression patterns and distinct sensitivity to G-protein-mediated inhibition. Therefore, we sought to determine whether selective association with MAP1B-LC1 might underlie functional differences between e37 splice isoforms of $Ca_V2.2$ channels. We thus performed co-immunoprecipitation assays using lysates from HEK-293 cells transiently expressing the $Ca_V2.2e37a$ and $Ca_V2.2e37b$ isoforms along with $Ca_V\beta_3$ and $Ca_V\alpha_2\delta$-1 auxiliary subunits as well as the full-length MAP1B-LC1 protein. In these experiments both recombinant channel complexes co-immunoprecipitated with MAP1B-LC1 and also with the E2 enzyme of the UPS UBE2L3 (**Fig. 1**). It should be noted, however, that in these IP assays 2 bands can be observed close to the molecular weight of MAP1B-LC1. The upper band may correspond to the MAP1B-LC1 protein, while the lower band may be the result of an unspecific interaction, since it can be also seen in the lane corresponding to the irrelevant antibody (**Fig. 1B**, left panel).

In order to investigate how this interaction influences the final surface density of $Ca_V2.2$ channels, we next determined the relative contribution of membrane insertion or recycling on the MAP1B-LC1-mediated regulation of the channels. This was done by using the cell-permeant inhibitors of vesicle trafficking brefeldin A (BFA),[9] and the dynamin inhibitor dynasore.[10] HEK-293 cells transiently transfected with $Ca_V2.2e37a/Ca_V\beta_3/Ca_V\alpha_2\delta$-1 channels in presence or absence of MAP1B-LC1 were pretreated for 6 h (48 h post-transfection) with the inhibitors and subjected to patch-clamp recording. Whole-cell Ba^{2+} currents through recombinant Ca^{2+} channels showed that BFA had no apparent effect on the MAP1B-LC1-mediated inhibition of $Ca_V2.2$ channel functional expression, suggesting that the forward trafficking of the channels was not altered. In contrast, dynasore, a clathrin-mediated endocytosis blocker significantly prevented the effect of MAP1B-LC1 on $Ca_V2.2$ channel surface expression (**Fig. 2B**). Therefore, these results point to a dynamin-dependent endocytosis mechanism that underlies the MAP1B-LC1-mediated $Ca_V2.2$ channel regulation.

To determine whether this mechanism is also valid for recombinant P/Q-type ($Ca_V2.1$) channels, a series of experiments using specific antibodies were then performed to study whether MAP1B-LC1 and the ubiquitin E2 conjugase UBE2L3 could be immunoprecipitated in samples from transfected HEK-293 cells. As shown in **Fig. 3A**, immunoprecipitation with $Ca_V2.1\alpha_1$ antibodies resulted in a band between the size markers of 15 and 20 kDa for UBE2L3 (expected size 17 kDa) and of ~36 kDa in the case of MAP1B-LC1. These data indicate that the ubiquitination/degradation mechanism triggered by MAP1B-LC1 might be conserved among N-type and P/Q-type channels. Next, we sought to determine whether MAP1B-LC1 and UBE2L3 may also interact with, and possible modulate the expression of native P/Q-type Ca_V channels. Polyclonal anti-$Ca_V2.1\alpha_1$ antibodies were used to immunoprecipitate either UBE2L3 or MAP1B-LC1 proteins in extracts from rat brain. Probing the immunoprecipitates for the presence of both proteins revealed immunoreactive bands (**Fig. 3B**). In contrast, UBE2L3 immunoreactivity was not detected in

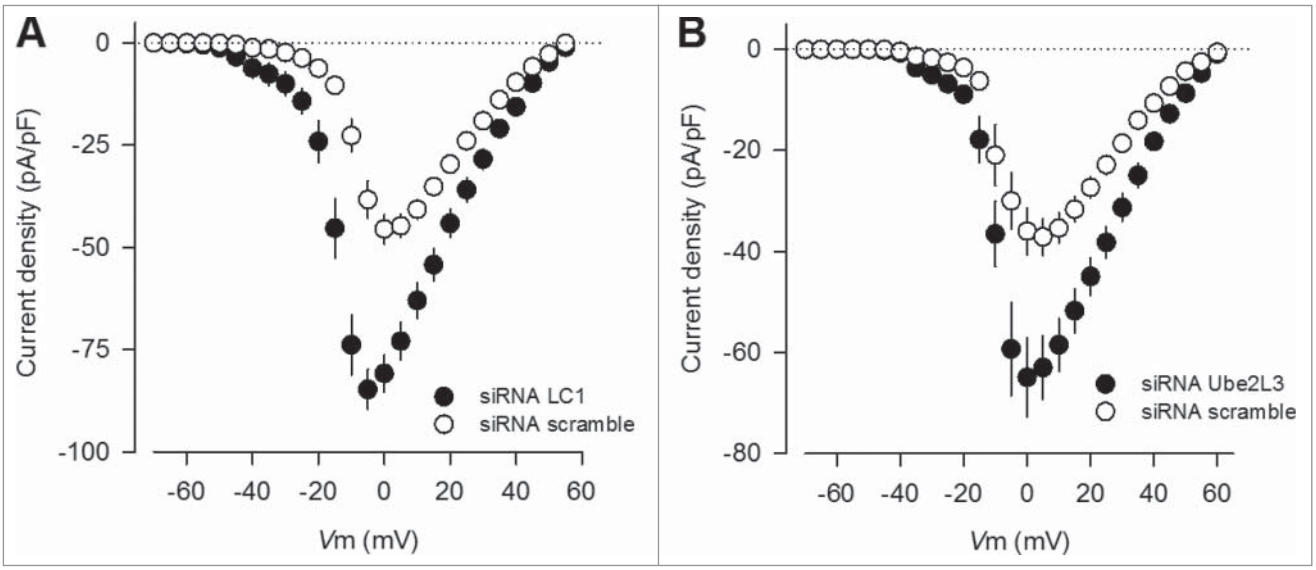

Figure 2. MAP1B-LC1-mediated regulation involves an internalization of the channels through a dynamin and clathrin-dependent pathway. The graphs show the average current densities (pA/pF) as a function of voltage in HEK-293 cells transfected with $Ca_V2.2\alpha_1/Ca_V\beta_3/Ca_V\alpha_2\delta$-1 channels in the absence (control) and the presence of LC1 ($n = 9$). In a subset of LC1 co-expressing cells, recordings were performed in the presence of BFA (**A**) or dynasore (**B**); $n = 14$ and 12 recorded cells, respectively. Note that the dynamin inhibitor dynasore prevents the inhibitory actions of LC1 on channel current functional expression.

immunoprecipitates using anti-$Ca_V3\alpha_1$ LVA channel subunit or GFP antibodies, both in native and heterologously expressed channels (**Figs. 3C and D**), indicating the HVA channel selective nature of the observed MAP1B-LC1/UBE2L3 co-association.

Finally, to further support the model of the important role of MAP1B-LC1 and UBE2L3 in Ca_V2 channel ubiquitination, we next tested whether knockdown of these proteins were capable of modifying Ca^{2+} currents in neonatal mouse dorsal root ganglion (DRG) sensory neurons, where N-type ($Ca_V2.2$) channels are prominently expressed.[11,12] We measured whole-cell currents 48 h after transfection of specific siRNAs targeting MAP1B-LC1 or UBE2L3. For these experiments fluorescently labeled siRNAs were used to identify the transfected cells. The results show that current amplitude from DRG neurons with expression of the scramble siRNA was not different from non-transfected cells, indicating that the transfection procedure did not affect channel activity (not shown). In contrast, current density after MAP1B-LC1- or UBE2L3-targeted knockdown in neurons was significantly different from that in control neurons transfected with scrambled siRNA (**Figs. 4A and B**). These findings suggest that UBE2L3 interacts with HVA channels to regulate their density at the plasma membrane. Moreover, they reinforce the idea that the effect of UBE2L3 requires the presence of the MAP1B-LC1 protein.

In summary, our findings suggest that $Ca_V2.2$ and possibly other Ca_V2 channels are potential targets of the ubiquitin-proteasome system (UPS), and suggest a model according to which these channels form a molecular complex with the MAP1B-LC1 protein (**Fig. 5**). Through MAP1B-LC1, Ca_V2 channels bind to the ubiquitin conjugase UBE2L3 favoring channel degradation in the proteasome. In this context, it is conceivable that MAP1B-LC1 may function as a scaffold protein to increase UBE2L3-mediated channel ubiquitination. It is interesting to note that the density of Ca_V2 channels was not affected by MAP1B-LC1 upon blocking the endocytotic machinery. This may suggest that the MAP1B-LC1/UBE2L3 complex mediated decrease in plasma membrane density of the channels occurs via a dynamin-mediated pathway. Finally, given that Ca_V2 channels are expressed throughout the nervous system, our results suggest that they might be under tonic control by the UPS in most neurons. This is of particular physiological relevance since it has been shown that the inhibition of the UPS may lead to increases in synaptic efficacy and neurotransmission.[13]

Materials and Methods

Cell culture and cDNA clone transfection

HEK-293 cells (ATCC) were grown in DMEM-high glucose medium supplemented with 10% horse serum, 2 mM L-glutamine, 110 mg/L sodium pyruvate, 100 U/L penicillin, and 100 μg/L streptomycin. Cell culture was maintained at 37°C in 5% CO_2, 95% air humidified atmosphere. Cells were transfected using Lipofectamine Plus reagent (Invitrogen) as previously described.[5] HEK-293 cells were seeded onto 35 or 60 mm dishes and transfected using 2 or 4 μg respectively, of each plasmid cDNA encoding N-type channel pore-forming subunit $Ca_V2.2\alpha_1$ (37a or 37b splicing isoforms), $Ca_V2.1\alpha_1$, $Ca_V\beta_3$, $Ca_V\alpha_2\delta$-1, $Ca_V3.1\alpha_1$-YFP, $Ca_V3.2\alpha_1$-GFP or MAP1B-LC1. Transfected cells were identified by adding a plasmid cDNA encoding the green fluorescent protein to the transfection mixture.

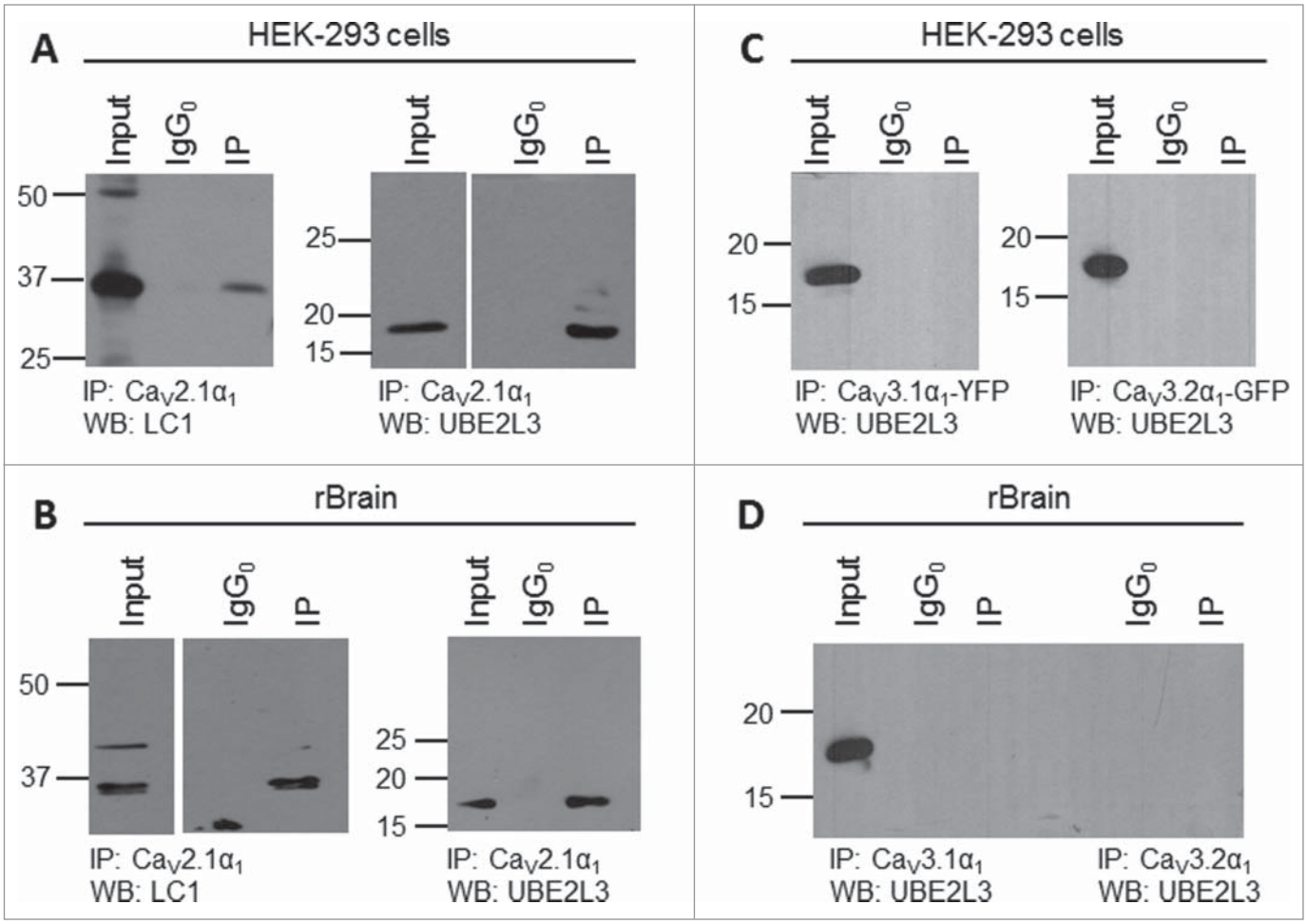

Figure 3. The protein complex UBE2L3-MAP1B-LC1-$Ca_V\alpha_1$ is conserved between HVA channels, but not with LVA calcium channels. Proteins from HEK-293 cells co-transfected with LC1-myc and P/Q-type channels ($Ca_V2.1\alpha_1/Ca_V\beta_3/Ca_V\alpha_2\delta$) were immunoprecipitated with the anti-$Ca_V2.1\alpha_1$ or control antibodies and the bound protein was analyzed by Western blot using an antibody against UBE2L3 and LC1 (**A**). Positive interactions are observed in both cases ($n = 3$). The irrelevant control (IgG_0) was a rabbit anti-Pet antibody. Likewise, proteins from adult rat brain lysates were immunoprecipitated with the anti-$Ca_V2.1\alpha_1$ (**B**), or anti-$Ca_V3.1\alpha_1$ and anti-$Ca_V3.2\alpha_1$ (**C and D**) or control antibodies and the bound protein was analyzed by Western blot using antibodies against UBE2L3 or LC1 ($n = 2$–4). Note that Ca_V3 channels do not interact with UBE2L3. The irrelevant controls (IgG0) were rabbit anti-Pet, chicken anti-HA, and mouse anti-HA antibodies, respectively.

Protein extraction

Cells were detached from culture dishes, washed with phosphate-buffered saline (PBS; pH 7.4), and lysed in single-detergent lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and Complete 1×; Roche Applied Science). Rat brain proteins were lysed in RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.5 mM PMSF and Complete 1×). Protein concentration was determined using the bicinchoninic acid assay.

Co-immunoprecipitation and Western blot

Co-immunoprecipitation assays were performed using rat brain proteins or lysates from transfected HEK-293 cells. One mg of proteins was incubated with 3 μg of specific antibodies $Ca_V2.1\alpha_1$ and $Ca_V2.2\alpha_1$ (KP Campbell, U of Iowa, USA), GFP (Aves lab, GFP-1020), $Ca_V3.1\alpha_1$ (Alomone, ACC-021), $Ca_V3.2\alpha_1$ (Millipore, AB9826), HA (Aveslab, ET-HA100), HA (Millipore, 32–6700), Pet (F. Navarro, Cinvestav, Mexico) and with 20 μl of recombinant Protein G (rProtein G) Agarose (Invitrogen) at 4°C overnight. The immunoprecipitated proteins were recovered by centrifugation (5 min at 13,000 rpm) and washed 3 times with wash buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS 0.5 mM PMSF) and 2 times with PBS. Samples were eluted into 30 μl of protein loading buffer. Fifty μg of protein samples were used as inputs. Proteins were resolved in 16% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with 5% nonfat dry milk in Tris-buffered saline Tween 20 (TBST; 100 mM Tris-Cl, 0.9% w/v NaCl, 0.2% Tween 20, pH 7.5), membranes were

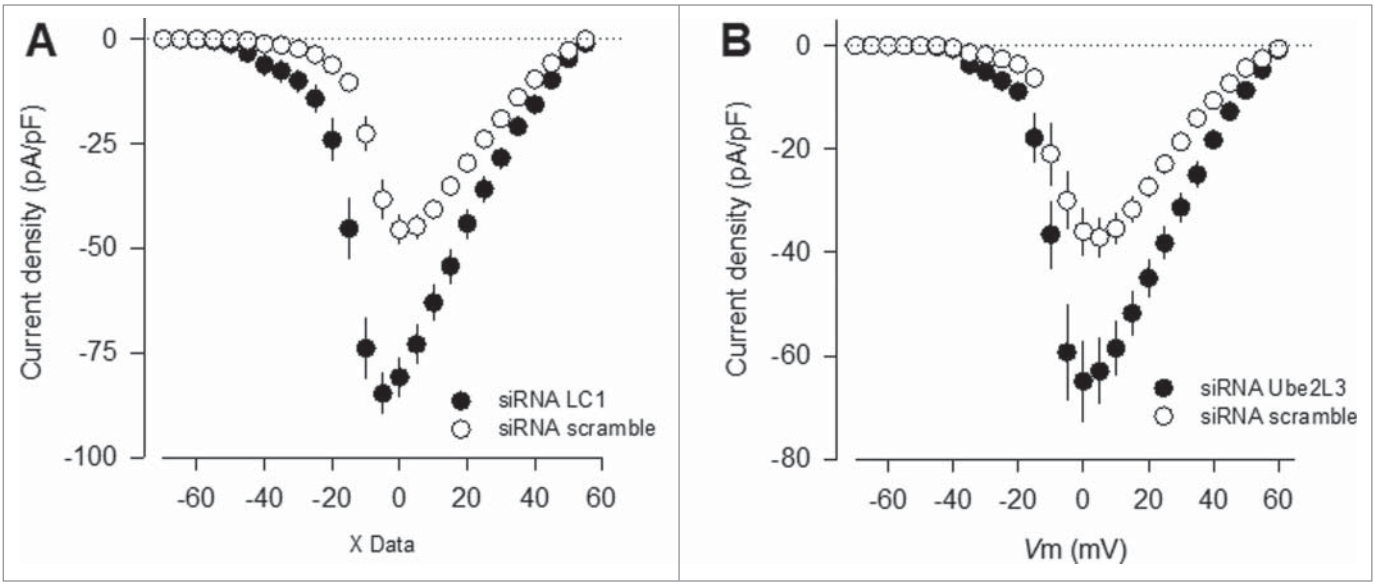

Figure 4. Knockdown of endogenous MAP1B-LC1 or UBE2L3 alters $Ca_V2.2$ channel functional expression. The graphs compare the average current densities (pA/pF) as a function of voltage in DRG neurons transfected with a siRNA scramble or specific siRNAs targeting MAP1B-LC1 (**A**) or UBE2L3 (**B**); $n = 13$ and 15 recorded cells, respectively.

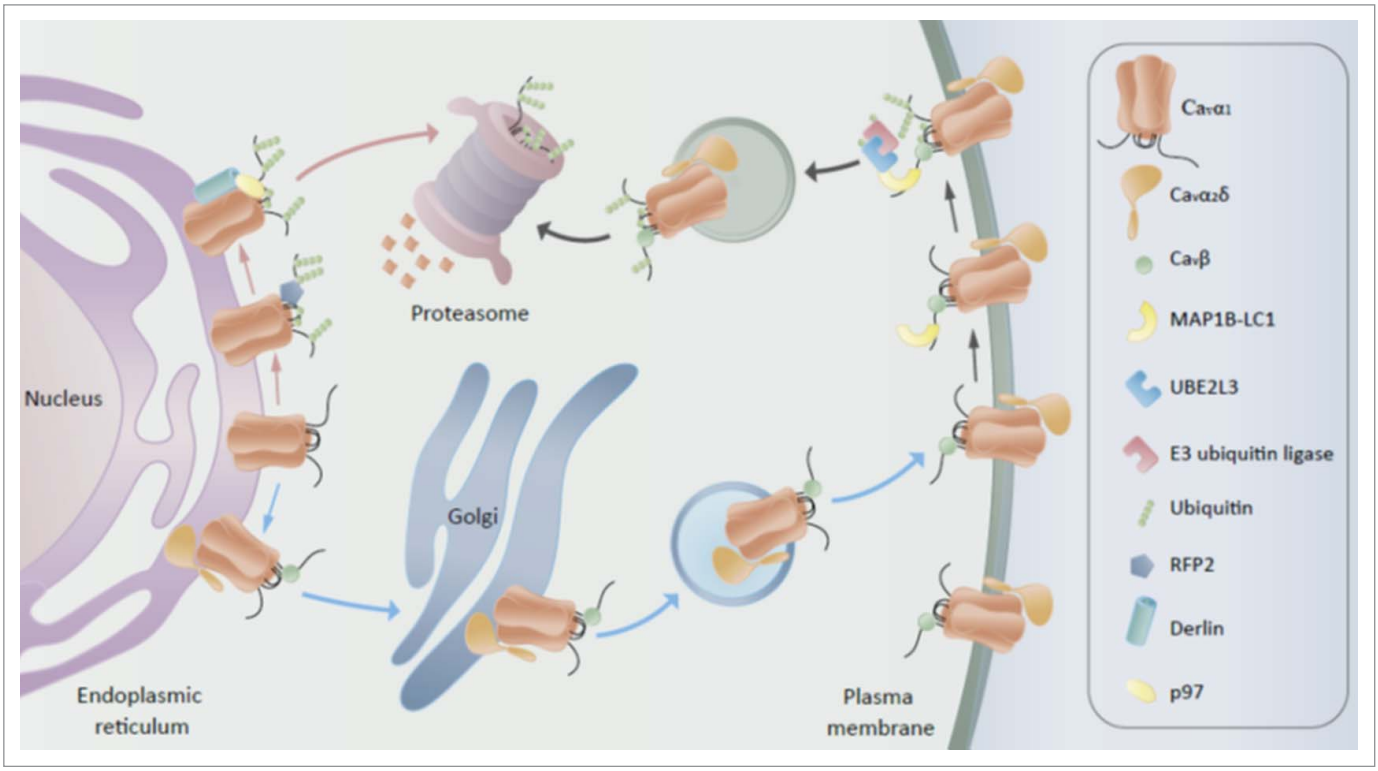

Figure 5. Model for MAP1B-LC1/UBE2L3-mediated Ca_V2 channel regulation. MAP1B-LC1 is known to bind to the C-terminal of the $Ca_V2.2$ and possibly the $Ca_V2.1$ α_1 pore-forming subunits and can interact directly with UBE2L3, which leads to channel poly-ubiquitination. After ubiquitination the Ca_V2 channel complexes are internalized and degraded in the proteasome consequently leading to a reduction of functional channels at the cell membrane. Alternatively, previous studies have shown that the ubiquitin ligase RFP2 may promote the L-type Ca_V channel poly-ubiquitination leading to association with derlin and protein p97 important elements of the endoplasmic reticulum-associated protein degradation (ERAD) complex. Poly-ubiquitinated channels are then subjected to proteasomal degradation. In this model, the $Ca_V\beta$ auxiliary subunit prevents RPF2 association and channels are consequently trafficked to the plasma membrane.[15]

incubated overnight with primary antibodies MAP1B (Santa Cruz, ET-HA100) or UBE2L3 (Abcam, ab37913) in TBST. Membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies diluted in TBST with 5% nonfat dry milk and developed with Millipore Immobilion Western Chemiluminescent HRP Substrate (ECL).

Electrophysiology

DRG cells were isolated from 5- to 7-day-old BALB/c mice. Dissociated neurons were maintained in neurobasal medium supplemented with B27 (1X), N2 (1X), Glutamax (1X), antibiotic-antimicotic (1X) and sodium pyruvate (110 mg/L). Cells were plated on poly-L-lysine (0.05%)-precoated glass coverslips placed into 35-mm culture plates and subjected to electrophysiological recording performed according to the whole cell configuration of the patch clamp technique,[12,14] 48 h after transfection of fluorescently labeled siRNAs targeting MAP1B-LC1 or UBE2L3. Currents were recorded from using the following extracellular solution (in mM): 120 TEA-Cl, 10 $BaCl_2$, 10 HEPES, and 10 glucose (pH 7.4). The intracellular solution consisted of (in mM) 135 CsCl, 2 $MgCl_2$, 10 HEPES, 4 MgATP, 0.01 GTP, and 10 EGTA (pH 7.1). Brefeldin A and Dynasore were supplied by Sigma and were used at 50 ng/ml and 40 μM, respectively.

siRNA labeling and transfection

siRNAS obtained from Sigma (GUUCGUAAACAUUACCAAA [dT] and CAGUGAAACAGGUCAAACU [dT] for MAP1B-LC1 and CGUUUC-CUGUGCCAACACU[dT] and CACC-CUAACAUCGAUGAGA [dT] for UBE2L3) were labeled using Silencer siRNA labeling kit Cy3 (Ambion) following manufacturer's instructions. Neurons were transfected using Lipofectamine 2000 (Invitrogen) with 0.5 μg of each labeled siRNA. Neurons transfected with universal negative control #1 siRNA (Sigma) were used as control.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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