BACKGROUND: The turnover of cardiac ion channels underlying action potential duration is regulated by ubiquitination. Genome-wide association studies of QT interval identified several single-nucleotide polymorphisms located in or near genes involved in protein ubiquitination. A genetic variant upstream of LITAF (lipopolysaccharide-induced tumor necrosis factor) gene prompted us to determine its role in modulating cardiac excitation.

METHODS: Optical mapping was performed in zebrafish hearts to determine Ca²⁺ transients. Live-cell confocal calcium imaging was performed on adult rabbit cardiomyocytes to determine intracellular Ca²⁺ handling. L-type calcium channel (LTCC) current (I_{Ca,L}) was measured using whole-cell recording. To study the effect of LITAF on Cav1.2 (L-type voltage-gated calcium channel 1.2) channel expression, surface biotinylation, and Westerns were performed. LITAF interactions were studied using coimmunoprecipitation and in situ proximity ligation assay.

RESULTS: LITAF knockdown in zebrafish resulted in a robust increase in calcium transients. Overexpressed LITAF in 3-week-old rabbit cardiomyocytes resulted in a decrease in I_{Ca,L} and Cavα1c abundance, whereas LITAF knockdown increased I_{Ca,L} and Cavα1c protein. LITAF-overexpressing decreases calcium transients in adult rabbit cardiomyocytes, which was associated with lower Cavα1c levels. In tsA201 cells, overexpressed LITAF downregulated total and surface pools of Cavα1c via increased Cavα1c ubiquitination and its subsequent lysosomal degradation. We observed colocalization between LITAF and LTCC in tsA201 and cardiomyocytes. In tsA201, NEDD (neural precursor cell expressed developmentally downregulated protein) 4-1, but not its catalytically inactive form NEDD4-1-C867A, increased Cavα1c ubiquitination. Cavα1c ubiquitination was further increased by coexpressed LITAF and NEDD4-1 but not NEDD4-1-C867A. NEDD4-1 knockdown abolished the negative effect of LITAF on I_{Ca,L} and Cavα1c levels in 3-week-old rabbit cardiomyocytes. Computer simulations demonstrated that a decrease of I_{Ca,L} current associated with LITAF overexpression simultaneously shortened action potential duration and decreased calcium transients in rabbit cardiomyocytes.

CONCLUSIONS: LITAF acts as an adaptor protein promoting NEDD4-1–mediated ubiquitination and subsequent degradation of LTCC, thereby controlling LTCC membrane levels and function and thus cardiac excitation.
on channel proteins are regulated by various types of posttranslational modifications. In most cases, ubiquitination acts as a signal for endocytosis of the ion channels, which are subsequently degraded through lysosomal or proteasomal-dependent pathways. Ubiquitination also occurs at the exit of endoplasmic reticulum and the trans-Golgi-network.\textsuperscript{1–4} Several genome-wide association studies for loci that modify the QT interval and the risk for sudden cardiac death\textsuperscript{5,6} have identified 3 single-nucleotide polymorphisms located in or near genes involved in protein ubiquitination: (1) the RNF207 (Ring finger protein 207) ubiquitin ligase\textsuperscript{7,8}; (2) RFFL (Ring finger and FYVE [Fab 1, YOTB, Vac 1, and EEA1]-like domain-containing E3 ubiquitin-protein ligase);\textsuperscript{9,10} and (3) LITAF (lipopolysaccharide-induced tumor necrosis factor), a regulator of endosomal trafficking\textsuperscript{11–13} and inflammatory cytokines,\textsuperscript{14,15} and an adapter molecule for members of the NEDD4 (neural precursor cell expressed developmentally down-regulated protein 4)-like family of E3 ubiquitin ligases.\textsuperscript{16,17} The genetic variant rs8049607 (Figure 1 in the Data Supplement) was associated with a modest QT-interval–prolongation effect (1.2 ms, \(P=5\times10^{-15}\)).\textsuperscript{5,6} It is ≈41 kb upstream of the LITAF start codon\textsuperscript{18} and located within an intergenic enhancer region.\textsuperscript{19} Furthermore, data from expression quantitative trait loci analyses showed that rs8049607 was also associated with reduced LITAF mRNA transcript levels in the left ventricle (Figure 1 in the Data Supplement).\textsuperscript{20}

LITAF is a mediator of local and systemic inflammatory responses\textsuperscript{15} and is locally upregulated in many inflammatory diseases, such as Crohn disease and ulcerative colitis.\textsuperscript{21} Notably, whole-body LITAF deletion diminished experimental endotoxic shock and inflammatory arthritis in mice.\textsuperscript{15} Importantly, loss-of-function mutations in LITAF cause Charcot-Marie-Tooth disease, an inherited peripheral neuropathy. These mutations\textsuperscript{22,23} are clustered around the hydrophobic region required for membrane localization and cause mislocalization and impaired endosome-to-lysosome trafficking of membrane proteins\textsuperscript{11,24} (Figure 1A).

Although there has been some controversy as to the activity of LITAF as a transcription factor,\textsuperscript{14,21,25} many studies have established its functional role in endosomal trafficking and multivesicular body formation.\textsuperscript{11,12,26} Indeed, LITAF interacts with members of the ESCRT (endosomal sorting complex required for transport), including TSG101 (tumor susceptibility gene 101) (via LITAF’s tetrapeptide motif, P[S/T]AP; Figure 1A) and STAM1 (signal transducing adaptor molecule 1) (physical interaction with LITAF), recruits them to the early endosomal membrane, and controls endosome-to-lysosome trafficking and exosome formation.\textsuperscript{11} The N-terminus of LITAF contains 2 PXY motifs (Figure 1A), which are important for interacting with members of the NEDD4 family of HECT (homologous to the E6-AP carboxyl terminus) domain ubiquitin ligases via their WW domains.\textsuperscript{12,27} Based on the genome-wide association studies’ findings\textsuperscript{5,6} and LITAF’s functional role in endosome-to-lysosome trafficking, we hypothesized that LITAF is a candidate for regulation of cardiac excitation, likely acting as an effector of ion channel complex trafficking or degradation through lysosomes. Therefore, we set out to investigate the possible role of LITAF in the regulation of ion channels in zebrafish heart and rabbit cardiomyocytes. In this study, we present data that support a role for LITAF in modulating membrane abundance and function of voltage-gated L-type calcium channels (LTCCs) via the ubiquitin ligase NEDD4-1 in the heart.

**METHODS**

We declare that all supporting data are available within the article (and its Data Supplement). All animal experiments and procedures were approved by the Rhode Island Hospital Institutional Animal Care and Use Committee. Experiments performed on zebrafish (*Danio rerio*) are in accordance with animal protocols approved by the Harvard Medical School Institutional Animal Care and Use Committee. An expanded Methods section is found in the Data Supplement.

**RESULTS**

**Genetic Knockdown of LITAF in Larval Zebrafish Hearts Affects Calcium Transients**

In an effort to delineate the effects of cardiac LITAF in vivo, we designed a morpholino oligomer to the zebrafish ortholog (LITAF; Figure 1A) targeting the ATG. In initial dose-ranging studies, there was no evidence of cardiac or systemic toxicity (data not shown). Electrophysiological studies of the ventricular myocardium revealed that a LITAF knockdown in zebrafish larvae caused a robust increase in the amplitude of [Ca\textsuperscript{2+}], transients compared with control morphants at 48 hours post-fertilization (Figure 1B through 1E). These data suggest that LITAF modulates Ca\textsuperscript{2+} handling in zebrafish. Because the phasic [Ca\textsuperscript{2+}], transient in zebrafish largely depends on Ca\textsuperscript{2+} influx through transmembrane Ca\textsuperscript{2+} channels,\textsuperscript{28} we reasoned that LITAF may be critical to regulating voltage-gated LTCC in the heart.

**Effect of LITAF Overexpression on Ca\textsuperscript{2+} Cycling in Adult Rabbit Cardiomyocytes**

Because our in vivo observations in zebrafish embryos showed an increase in Ca\textsuperscript{2+} transients in LITAF morphants (Figure 1B through 1E), we expected LITAF to also interfere with intracellular Ca\textsuperscript{2+} handling in rabbit cardiomyocytes. Therefore, we utilized cultured adult rabbit cardiomyocytes, which in our hands and in agreement with Tian et al\textsuperscript{29} largely preserve t-tubules in culture (data not shown), to study the effect of adeno-virally expressed LITAF on cardiac Ca\textsuperscript{2+} cycling.
We performed live-cell confocal imaging and measured the amplitude of electrically evoked Ca\textsuperscript{2+} transients in cells overexpressing LITAF or GFP (green fluorescence protein) as control (Figure 2A and 2B). To determine the sarcoplasmic reticulum-Ca\textsuperscript{2+} load and assess NCX (Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger) function, 20 mmol/L caffeine was applied at the end of the experiments. We observed that LITAF overexpression in adult rabbit cardiomyocytes significantly decreased the amplitude of Ca\textsuperscript{2+} transients (Figure 2A and 2B). This decrease was paralleled by a decrease in sarcoplasmic reticulum Ca\textsuperscript{2+} content assessed by rapid application of 20 mmol/L caffeine. There were no changes in fractional release of Ca\textsuperscript{2+} (Figure 2B). NCX activity calculated by measuring the rate of decay of caffeine-induced Ca\textsuperscript{2+} transients was not different between the groups (Figure 2B). No change in SERCA2 (sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase 2) activity was found as well (Figure 2B), which was calculated via a derived rate of decay, subtracting the rate of decay of pacing-induced Ca\textsuperscript{2+} transients from the rate of decay of caffeine-induced Ca\textsuperscript{2+} transients. This is corroborated by Western blot data showing no significant changes in total levels of SERCA2, NCX, calsequestrin 2, or serine 16-phosphorylated phospholamban upon LITAF overexpression in cardiomyocytes (Figure II in the Data Supplement). Together, these data indicate that the reduction in Ca\textsuperscript{2+} transients is likely due to reduced Ca\textsuperscript{2+} influx, which is supported by a significant down-regulation of the total pool of Cav\textsubscript{α1c} in LITAF-overexpressing cells (Figure 2C and 2D).

**LITAF Modulates I\textsubscript{Ca,L} and Cav\textsubscript{α1c} Protein in 3-Week-Old Cardiomyocytes**

To study any effect of LITAF on Cav\textsubscript{α1c} and LTCC current (I\textsubscript{Ca,L}) in detail, we switched to cultured 3-week-old...
Figure 2. Attenuation of Ca\(^{2+}\) transients and Cav\(\alpha_{1c}\) (L-type calcium channel alpha-1C subunit) abundance by LITAF (lipopolysaccharide-induced tumor necrosis factor) in adult rabbit cardiomyocytes.

Cardiomyocytes were transduced with adenovirus expressing GFP (green fluorescence protein) or HA (hemagglutinin)-LITAF. A, Representative confocal line-scan images and corresponding fluo-3 F/F\(_0\) time-dependent profiles at 1 Hz. B, Histograms depict mean data from Ca\(^{2+}\) transient amplitudes (GFP, 1.78±0.16 vs LITAF, 1.2±0.13 ∆F/F\(_0\)), caffeine transient amplitudes (GFP, 2.8±0.15 vs LITAF, 2.1±0.19 ∆F/F\(_0\)), fractional release and rates of Ca\(^{2+}\) removal by NCX (Na+/Ca\(^{2+}\) exchanger; \(k_{sa}\)) and SERCA2 (sarcoplasmic reticulum Ca\(^{2+}\)-ATPase 2; \(k_{SR}\)). Student t test, \(P<0.05\) (2-3 heart preparations). C, Adult rabbit cardiomyocytes lysates were probed with anti-Cav\(\alpha_{1c}\), anti-HA, and anti-GAPDH to indicate Cav\(\alpha_{1c}\), exogenous LITAF and GAPDH (loading control) protein levels. D, Respective change in Cav\(\alpha_{1c}\) abundance, normalized to GAPDH (n=5 animals, performed in triplicate; mean±SEM). Student t test, \(P<0.05\).
rabbit cardiomyocytes. We have recently developed this model system to investigate various ion channels underlying action potential duration (APD; data not shown). For example, these cells exhibit stable $I_{Ca,L}$ current after 48 hours in culture (Figure 3A and 3B). The cells were transduced with adenovirus encoding GFP (green fluorescence protein) or HA (hemagglutinin)-LITAF for 48 h. A, Left, voltage steps to measure I-V for $I_{Ca,L}$ in cardiomyocytes. B, Voltage dependence of $I_{Ca,L}$ current density in cardiomyocytes transduced with GFP or LITAF (cells from 5 animals; mean±SEM; Student t test, P<0.05). C, Protein levels of Cavα1c, HA-LITAF, and tubulin (left). Respective change in Cavα1c abundance, normalized to tubulin (n=5 animals, performed in triplicate; mean±SEM). Student t test, P<0.05 (right). D, Current-voltage relationships of $I_{Ca,L}$ peak currents for baseline conditions from cells expressing scrambled shRNA or short hairpin RNA (shRNA) against endogenous LITAF (cells from 5 animals; mean±SEM; Student t test, P<0.05). E, Protein levels of Cavα1c, total LITAF, and tubulin (left). Respective changes in Cavα1c and LITAF abundance, normalized to tubulin (n=5 animals, performed in triplicate; right).

Figure 3. Control of LTCC current ($I_{Ca,L}$) and Cavα1c (l-type calcium channel alpha-1C subunit) protein levels by LITAF (lipopolysaccharide-induced tumor necrosis factor) in 3-wk-old cardiomyocytes.

Cardiomyocytes were transduced with adenovirus encoding GFP (green fluorescence protein) or HA (hemagglutinin)-LITAF for 48 h. A, Left, voltage steps to measure I-V for $I_{Ca,L}$ in cardiomyocytes. B, Voltage dependence of $I_{Ca,L}$ current density in cardiomyocytes transduced with GFP or LITAF (cells from 5 animals; mean±SEM; Student t test, P<0.05). C, Protein levels of Cavα1c, HA-LITAF, and tubulin (left). Respective change in Cavα1c abundance, normalized to tubulin (n=5 animals, performed in triplicate; mean±SEM). Student t test, P<0.05 (right). D, Current-voltage relationships of $I_{Ca,L}$ peak currents for baseline conditions from cells expressing scrambled shRNA or short hairpin RNA (shRNA) against endogenous LITAF (cells from 5 animals; mean±SEM; Student t test, P<0.05). E, Protein levels of Cavα1c, total LITAF, and tubulin (left). Respective changes in Cavα1c and LITAF abundance, normalized to tubulin (n=5 animals, performed in triplicate; right).
knockdown efficiency of LITAF was ≈60% (Figure IVB in the Data Supplement) compared with cells expressing scrambled control RNA. Lowered LITAF levels resulted in a significant, 3.5-fold, increase in total Cav\(_{\alpha 1c}\) abundance (Figure IVB in the Data Supplement). Finally, quantitative polymerase chain reaction showed no changes in mRNA levels of LTCC in neonatal rabbit cardiomyocytes overexpressing LITAF, ruling out transcriptional effects of LITAF on LTCC (Figure IVC in the Data Supplement).

**LITAF Has No Significant Effect on the Major Repolarizing K\(^+\) Current, \(I_{Ks}\) (delayed rectifier potassium current) in 3wRbCM**

The aforementioned genome-wide association studies\(^5,6\) implied a role for LITAF in QT interval and, therefore, APD regulation. Interestingly, we have previously shown that 2 other genes identified in these studies, the RING finger ubiquitin ligases RNF207 and RFFL, affect a major repolarizing current in larger animals, (viz \(I_{Ks}\))\(^7,9\). Therefore, we set out to look for LITAF-dependent effects on this current. To this end, 3wRbCM were transfected with plasmids for Cav\(_{\alpha 1c}\), (L-type calcium channel alpha-1C subunit) Cav\(_{\beta 3}\), and Cav\(_{\alpha 2\delta-1}\) to reconstitute functional LTCC, GFP (green fluorescence protein), or HA (hemagglutinin)-tagged LITAF. Cell-surface protein levels were determined by biotinylation: cell-surface proteins were biotinylated using sulfo-NHS-SS-biotin, purified with neutravidin beads from total cell lysates, subjected to SDS-PAGE and blotted onto a nitrocellulose membrane. A representative Western blot shows total protein levels of Cav\(_{\alpha 1c}\), Cav\(_{\beta 3}\), Cav\(_{\alpha 2\delta-1}\), HA-LITAF, and tubulin (left). Respective change in total Cav\(_{\alpha 1c}\) abundance, normalized to tubulin levels (n=5, performed in triplicate; mean±SEM). Student \(t\) test, \(P<0.05\) (right). B, A representative Western blot shows cell-surface protein levels of Cav\(_{\alpha 1c}\), Cav\(_{\beta 3}\), Cav\(_{\alpha 2\delta-1}\), TFR (transferrin receptor), total LITAF, and HA-LITAF (left). Respective changes in cell membrane protein levels of Cav\(_{\alpha 1c}\), Cav\(_{\beta 3}\), and Cav\(_{\alpha 2\delta-1}\) normalized to transferrin receptor levels (n=5, performed in triplicate; mean±SEM). Student \(t\) test, \(P<0.05\) (right).

**Physical and Functional Interaction Between LITAF and L-type Ca\(^{2+}\) Channels in tsA201 Cells**

To explore the mechanisms underlying LITAF inhibition of LTCC, we used a heterologous expression system, viz tsA201 cells, which are frequently used to study LTCC in vitro because they process the multisubunit complex correctly and efficiently.\(^{31}\) We transfected tsA201 cells with expression plasmids for Cav\(_{\alpha 1c}\), Cav\(_{\beta 3}\), and Cav\(_{\alpha 2\delta-1}\) to express functional LTCC and plasmid for HA-tagged LITAF or empty control plasmid. Cell-surface biotinylation was performed on transfected cells and protein levels of Cav\(_{\alpha 1c}\) in total lysates and cell-surface fractions determined (Figure 4A and 4B). Overexpression of LITAF resulted in a significant downregulation of both total and surface-membrane levels of Cav\(_{\alpha 1c}\).
In contrast, no changes in cell-surface levels of Cavβ3 and Cavα2δ-1 were observed (Figure 4A and 4B). To see whether the functional interaction between LITAF and LTCC is based on a physical interaction, we performed coimmunoprecipitations with extracts from tsA201 cells cotransfected with expression plasmids for all 3 LTCC subunits and HA-tagged LITAF. Extracts were incubated with HA antiserum or isotype control, and immunoprecipitates probed with anti-Cavβ3 (Figure 5A) or anti-Cavα1c (Figure 5B) antibody. Western blot analyses suggest that LITAF is found in a protein complex with Cavα1c, as well as Cavβ3. Furthermore, to confirm colocalization between endogenous LITAF and LTCC in rabbit cardiomyocytes, we performed in situ proximity ligation assay. Representative images are shown in Figure 5. Specificity of the assay was shown by the lack of staining using mouse anti-LITAF or rabbit anti-LTCC as negative controls (Figure 5C). Similarly, no signals were obtained omitting primary antibodies from the assay (Figure 5C). As a positive control for the assay, we used rabbit anti-Cavα2δ-1 and mouse anti-Cavα2δ-1 to detect endogenous Cavα2δ-1 (Figure 5C). Using the combination of rabbit anti-LITAF and mouse anti-Cavα1c, the appearance of puncta suggests colocalization between LITAF and Cavα1c in cardiomyocytes (Figure 5C). Similar results were obtained using mouse anti-LITAF and rabbit anti-Cavα1c (Figure 5C).

**LITAF-Dependent Regulation of Ubiquitination and Degradation of Cavα1c in tsA201 Cells**

Because ubiquitination plays an important role in all 3 major protein degradation pathways, we examined whether Cavα1c was ubiquitinated in a LITAF-dependent manner. We reconstituted LTCC by transfecting tsA201 cells cotransfected with plasmids for Cavα1c, Cavβ3, Cavα2δ-1, GFP, or HA-tagged LITAF using isotype control (lane 1) or HA antibody (lanes 2 and 3). A representative immunoblot against Cavβ3 shows an interaction between LITAF and the Cavβ3 subunit (IP; the asterisk indicates the heavy chain of the IP capture antibody). Also shown is the immunoprecipitated HA-LITAF protein. Input levels of Cavβ3, HA-LITAF, and tubulin are shown below. B, IP of lysates from tsA201 cells transfected with plasmids for Cavα1c, Cavβ3, Cavα2δ-1, GFP, or HA-tagged LITAF using HA antibody. A representative immunoblot against Cavα1c shows an interaction between LITAF and the Cavα1c subunit (IP). Also shown is the immunoprecipitated HA-LITAF protein. Input levels of Cavα1c, HA-LITAF, and tubulin are depicted below. C, Duo-link in situ proximity ligation assay using rabbit anti-LITAF and mouse anti-Cavα1c antibodies (alternatively mouse anti-LITAF and rabbit anti-Cavα1c antibodies) in 3wRbCM, which are amenable to proximity ligation assay and express detectable levels of LITAF and LTCC. Colocalization between molecules is indicated by red puncta. No puncta were detected in negative controls in which primary antibodies were omitted or only one antibody was used (rabbit anti-LITAF, mouse anti-Cavα1c, mouse anti-LITAF, or rabbit anti-Cavα1c antibodies). As positive control for the assay, a combination of rabbit polyclonal anti-Cavα2δ-1 and mouse monoclonal anti-Cavα2δ-1 was used to detect endogenous Cavα2δ-1. Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole) (blue).
cells with expression plasmids encoding Cavα1c, Cavβ3, Cavα2δ-1, HA-tagged ubiquitin, and Flag-tagged LITAF, or empty control plasmid. Using anti-HA antibody to pull down ubiquitinated protein from cell lysates, followed by immunodetection of Cavα1c, we observed a LITAF-dependent increase in the ubiquitination level of Cavα1c (Figure 6A, left). Although total Cavα1c abundance was significantly reduced upon coexpression of LITAF, no changes in total Cavβ3 and Cavα2δ-1 levels were seen (Figure 6A, right). Next, we treated cells expressing functional LTCC or LITAF or control plasmid with chloroquine or MG132 (N-benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal) to determine which protein degradation pathway is involved in LITAF-mediated Cavα1c downregulation. The lysosomal inhibitor chloroquine completely prevented the effect of LITAF on Cavα1c protein levels, whereas the proteasomal inhibitor MG132 did not impair downregulation of Cavα1c by LITAF (Figure 6B).

Because LITAF binds to the ubiquitin ligase NEDD4-1, recently implicated in LTCC downregulation, we looked for a possible functional interaction between LITAF and NEDD4-1 with respect to ubiquitination of LTCC. To this end, we transfected cells with plasmids for Cavα1c, Cavβ3, Cavα2δ-1, and HA-tagged ubiquitin. A representative immunoblot shows levels of ubiquitinated Cavα1c (left) and input levels of Cavα1c, Cavβ3, and Cavα2δ-1, Flag-tagged LITAF, and GAPDH (right). B, LITAF-mediated degradation of Cavα1c through lysosomes. Cells were transfected with plasmids for Cavα1c, Cavβ3, and Cavα2δ-1, GFP as control, or HA-tagged LITAF for 24 h and then treated with 10 µM chloroquine or 5 µM MG132 (N-benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal) for 20 h. Representative Western blots show total abundance of Cavα1c and tubulin of treated cells. C, IP of lysates from cells transfected with plasmids for Cavα1c, Cavβ3, and Cavα2δ-1, HA-tagged ubiquitin, GFP as control, NEDD4-1 (N4), NEDD4-1-C867A (N4mut), or Flag-tagged LITAF was performed with anti-HA antiserum. A representative immunoblot shows levels of ubiquitinated Cavα1c (top) and input levels of Cavα1c, Cavβ3, Cavα2δ-1, Flag-tagged LITAF, and GAPDH (bottom). D, Respective changes in the level of ubiquitinated Cavα1c, normalized to total Cavα1c (5 experiments, performed in duplicate, mean±SEM). Student t test, P<0.05.

Figure 6. LITAF (lipopolysaccharide-induced tumor necrosis factor)-mediated ubiquitination and degradation of Cavα1c (L-type calcium channel alpha-1c subunit) in tsA201 cells.
end, we transfected tsA201 cells with expression plasmids for HA-tagged ubiquitin, Cavα1c, Cavβ3, and Cavα2/δ-1, NEDD4-1, and tubulin in cells expressing scrambled control RNA or shRNA against endogenous NEDD4-1 (left; the asterisk indicates an unspecific band). Respective changes in NEDD4-1 and Cavα1c abundance, normalized to tubulin (n=5 animals, performed in triplicate; mean±SEM). Student t test, P<0.05 (right).

B. Current-voltage relationships of L-type calcium channel current (I_{Ca,L}) peak currents for baseline conditions from cells expressing GFP (green fluorescence protein) and shRNA against endogenous NEDD4-1 (control) or LITAF and NEDD4-1 shRNA. 

C. Three-week-old rabbit cardiomyocytes were transduced with adenovirus expressing scrambled RNA and LITAF (control) or LITAF and shRNA against endogenous NEDD4-1. Current-voltage relationships of I_{Ca,L} peak currents for baseline conditions from respective cells are depicted (cells from 5 animals; mean±SEM; Student t test, P<0.05).

**Figure 7.** NEDD (neural precursor cell expressed developmentally downregulated protein) 4-1-dependent downregulation of L-type calcium channel (LTCC) by LITAF (lipopolysaccharide-induced tumor necrosis factor) in 3-wk-old rabbit cardiomyocytes.

A. Protein levels of total Cavα1c (L-type calcium channel alpha-1c subunit), NEDD4-1, and tubulin in cells expressing scrambled control RNA or shRNA against endogenous NEDD4-1 (left; the asterisk indicates an unspecific band). Respective changes in NEDD4-1 and Cavα1c abundance, normalized to tubulin (n=5 animals, performed in triplicate; mean±SEM). Student t test, P<0.05 (right). 

B. Current-voltage relationships of LTCC current (I_{Ca,L}) peak currents for baseline conditions from cells expressing GFP (green fluorescence protein) and short hairpin RNA (shRNA) against endogenous NEDD4-1 (control) or LITAF and NEDD4-1 shRNA. 

C. Three-week-old rabbit cardiomyocytes were transduced with adenovirus expressing scrambled RNA and LITAF (control) or LITAF and shRNA against endogenous NEDD4-1. Current-voltage relationships of I_{Ca,L} peak currents for baseline conditions from respective cells are depicted (cells from 5 animals; mean±SEM; Student t test, P<0.05).
type, we used a physiologically detailed computational model of rabbit ventricular myocyte with membrane voltage coupled to spatially distributed subcellular Ca\(^{2+}\) dynamics. Details of the model are provided in the Data Supplement. We first determined the \(I_{\text{Ca,L}}\) conductances in the model that reproduces the \(I_{\text{Ca,L}}\) peak current versus voltage curves measured in voltage-clamp mode (Figure 3B) for myocytes with GFP and LITAF. We found that reducing the number of functional sarcolemmal LTCC from 4 to 2 in each calcium release unit, where LTCCs are colocalized with RyR2 (ryanodine receptor 2) Ca\(^{2+}\) release channels, reproduced the \approx 50%\) reduction of whole-cell \(I_{\text{Ca,L}}\) current with LITAF compared with GFP. The whole-cell current is the summation of LTCCs from \approx 16,000 calcium release units spatially distributed throughout the cell. Results in Figure 8A show that, with this 50% reduction in the total number of LTCCs, the model reproduces well the quantitative voltage-clamp measurements of Figure 3B. We then paced the myocytes at a 2.5 Hz in current-clamp mode for \(I_{\text{Ca,L}}\) conductances (total number of LTCCs) corresponding to GFP and LITAF. The results show a 50% decrease of \(I_{\text{Ca,L}}\) conductance, resulting from LITAF overexpression, significantly reduces both local Ca\(^{2+}\) release (confocal line-scan equivalent in Figure 8B) and whole-cell Ca\(^{2+}\) transient amplitude (Figure 8C). It also shortens APD from 206 ms with GFP to 182 ms with LITAF (Figure 8D). The decrease of APD appears relatively small in view of the large decrease of \(I_{\text{Ca,L}}\) current during the action potential plateau phase (Figure 8E). Examination of other currents reveals that a shift of NCX current towards reverse mode during the AP plateau partly counterbalances the effect of decreased \(I_{\text{Ca,L}}\) current on APD. This shift is associated with a decrease of steady-state intracellular Na\(^{+}\) concentration [Na\(^{+}\)] (10.8 mmol/L with GFP versus 9.4 mmol/L with LITAF). Reduced [Na\(^{+}\)] with LITAF then promotes forward mode NCX current (Figure 8F), thereby prolonging APD and partly counterbalancing the effect of \(I_{\text{Ca,L}}\) reduction on APD shortening. We also note that, on the contrary, the reduced Ca\(^{2+}\) transient amplitude with LITAF promotes reverse mode NCX, but this effect is not as significant as the shift towards forward mode caused by [Na\(^{+}\)] reduction. LITAF overexpression in 3wRbCM showed a similar (insignificant) APD shortening (mean ventricular APD90±SEM: LITAF 189±19 ms [n=21] versus GFP 224±15 ms [n=25], \(P=0.29\)). Power analysis determined that we would need 122 cells from each group to achieve statistically significant results. Of note, morpholino-mediated downregulation of LITAF in zebrafish resulted in APD prolongation that did not reach statistical significance (mean ventricular APD80±SEM: morphants 283±10 ms versus wild-type 247±9 ms, \(P=0.11\), n=7 and n=9).

**DISCUSSION**

LITAF regulates endosomal trafficking\(^{11-13}\) and inflammatory cytokines\(^{14,15}\) and acts as an adapter molecule for members of the NEDD4-like family of E3 ubiquitin ligases\(^{16,17}\). Sequence variation in LITAF (rs8049607) is associated with QT interval prolongation\(^{5,6}\) and reduced LITAF mRNA expression in the left ventricle (Figure I in the Data Supplement).\(^{20}\) The present study provides the first empirical evidence that LITAF exerts an inhibitory effect on the abundance and function of cardiac LTCC, in part, by controlling NEDD4-1 ubiquitin ligases.
Knockdown of LITAF in zebrafish larvae resulted in a robust increase in cardiomyocyte calcium transients on Fura-2 imaging. Overexpressed LITAF in LITAF in 3-week-old rabbit cardiomyocytes resulted in a decrease in ICa,L and Cavα1c protein levels, whereas a LITAF knockdown increased ICa,L and Cavα1c protein levels. We observed a decrease in calcium transients in LITAF-overexpressing adult rabbit cardiomyocytes, which was accompanied by lower Cavα1c abundance. In tsA201 cells, overexpressed LITAF downregulated the total and surface pools of Cavα1c via increased Cavα1c ubiquitination and its subsequent lysosomal degradation. Coimmunoprecipitation showed that LITAF formed a complex with LTCC. Furthermore, in situ proximity ligation assay indicated colocalization between LITAF and LTCC in cardiomyocytes. In tsA201 cells, NEDD4-1, but not its catalytically inactive form NEDD4-1ΔC867A, increased Cavα1c ubiquitination compared with control. Cavα1c ubiquitination was further increased by coexpressed LITAF and NEDD4-1 but not NEDD4-1ΔC867A. Knockdown of NEDD4-1 using shRNA abolished the negative effect of LITAF on ICa,L and Cavα1c protein levels in 3-week-old rabbit cardiomyocytes. Computer simulations demonstrated that a decrease of ICa,L current associated with LITAF overexpression simultaneously shortened APD and decreased Ca2+ transient amplitude in rabbit ventricular myocytes.

**LITAF: Tissue and Substrate Specificity**

Currently, we cannot rule out that LITAF/NEDD4-1-mediated downregulation of LTCC exists in other tissues, such as smooth muscle, somatodendritic neurons or endocrine cells, which also express Cavα1c at significant levels, as LITAF and NEDD4-1 are ubiquitously expressed proteins. Thus it is conceivable the reported single nucleotide polymorphism may also result in changes of ICa,L in noncardiac tissue. Similarly, LITAF may also negatively affect forward trafficking of other α1-subunit isoforms that require a β auxiliary subunit for surface expression, for example, Cav1.3 and Cav2.3, which are also expressed in the heart. In contrast, T-type calcium channels, which do not require a beta subunit for their function, are likely not controlled by LITAF. Further studies are warranted to explore these possibilities.

**NEDD4-1–Mediated Effects of LITAF on Cavα1c Protein Levels**

The NEDD4 family of HECT ubiquitin ligases contains 9 members, which are all expressed in the heart. Most studies have focused on NEDD4-1 and NEDD4-2, and a plethora of potential targets have been identified in vitro. Not surprisingly, various ion channels have been reported to be ubiquitinated by these ubiquitin ligases. For example, voltage-gated potassium (HERG) or sodium channels (Nav1.5) are ubiquitinated by NEDD4-2, resulting in their lysosomal degradation. Interestingly, a recent report by Rougier et al suggests that NEDD4-1 promotes downregulation of newly synthesized Cavα1c at the endoplasmic reticulum/Golgi level in tsA201 cells. Prompted by these findings, we looked into the possibility that LITAF-dependent downregulation requires NEDD4-1. Here, we present data that clearly show that upon knockdown of endogenous NEDD4-1, the effect of LITAF on ICa,L is completely abolished in cardiomyocytes (Figure 7B), thus confirming a hypothesized requirement of a NEDD4-1–dependent polyubiquitination of Cavα1c. Prior authors did not observe any NEDD4-1–dependent increase in Cavα1c ubiquitination, in contrast to our findings of increased ubiquitination level of Cavα1c by NEDD4-1 or LITAF (Figure 6A, 6C, and 6D). These differences likely reflect the discrete experimental conditions used. For example, to amplify the ubiquitination signal, we cotransfected tsA201 cells with an expression plasmid for HA-tagged ubiquitin. Additionally, we added 10 mmol/L N-ethylmaleimide and iodoacetamide to the lysis buffer to block reactive cysteines and thereby prevent deubiquitination of proteins by deubiquitinases during sample processing. Notably, a significant LITAF-dependent increase in the overall ubiquitination level of total protein isolated from neonatal rabbit cardiomyocytes was observed (data not shown). In agreement with the study by Rougier et al who reported that Cavβ2 was essential for NEDD4-1–mediated regulation of Cavα1c in tsA201 cells, we observed an absolute requirement for the accessory subunit Cavβ3 (or Cavβ2; data not shown) in LITAF-mediated downregulation of Cavα1c in the same cell line (data not shown). This is corroborated by our coimmunoprecipitation findings in tsA201 cells, demonstrating that LITAF was found in a protein complex with Cavα1c as well as Cavβ3 (Figure 5A and 5B) or Cavβ2 (data not shown) and by our in situ proximity ligation assay, which demonstrates colocalization between LITAF and LTCC in cardiomyocytes (Figure 5C).

As shown in Figure 6B, the lysosomal inhibitor chloroquine but not the proteasomal inhibitor MG132 blocked LITAF-dependent Cavα1c downregulation. Experiments in tsA201 cells (Figure 6A) also show Cavα1c polyubiquitination, indicated by the high molecular smear of ubiquitinated Cavα1c, in the presence of LITAF. Polyubiquitination linked through Lys48 or Lys11 generally leads to proteasomal degradation. In contrast, Lys63 linkages perform nondegradative roles (eg, cell signaling) but are also required for lysosomal degradation. Thus, we hypothesize that LITAF causes NEDD4-mediated Lys63 polyubiquitination of Cavα1c.

Contrary to the study by Rougier et al, we did not notice a LITAF-dependent downregulation of the accesso-
Regulation of NEDD4-1 Ubiquitin Ligase by LITAF

LITAF is a small zinc-binding monotopic membrane protein 49 found on the Golgi apparatus and multivesicular bodies. 12 It contains 2 N-terminal PXY motifs, which are required for physical interaction with WW domains of the HECT ubiquitin ligases NEDD4-1 12 and ITCH (itchy E3 ubiquitin protein ligase). 27 We demonstrated that LITAF interacts and colocalizes with LTCC subunits (Figure 5A through 5C) and forms a complex with LTCC. Previous studies have already observed colocalization of LITAF with NEDD4-1 on the Golgi apparatus 12 and control of Cav1c levels by NEDD4-1 at the endoplasmic reticulum/Golgi level. 23 Therefore, it is very likely that the LITAF-mediated ubiquitination of Cav1c is happening in the trans-Golgi-network (Figure VI in the Data Supplement). It is also conceivable that the physical adaptor LITAF promotes NEDD4-1 ubiquitination activity and recruits NEDD4-1 to its Cav1c substrate on trans-Golgi-network membranes (Figure VI in the Data Supplement). Such a scenario is reminiscent of the small NEDD4 family-interacting proteins, NDFIP1 (Nedd4 family interacting protein 1) and NDFIP2. 17 They contain cytoplasmic PXY motifs and are localized to the Golgi apparatus, endosomes, and multivesicular bodies. Previously, NDFIP1 was shown to recruit various NEDD4 ubiquitin ligases to membranes, promote autoubiquitination of these ubiquitin ligases by relieving their inherent autoinhibition, and induce substrate ubiquitination. 50 Similarly, a recent study by Kang et al 51 revealed that NDFIP1 recruits NEDD4-2 to the Golgi apparatus to mediate polyubiquitination-dependent degradation of HERG. It is interesting to note that both LITAF and NDFIP1 have been reported to promote exosome secretion, and both molecules could be detected in exosomes produced by cells overexpressing LITAF or NDFIP1, respectively. 26, 52

Transcriptional Effects of LITAF

Although LITAF has recently been shown to target intracellular membranes, 49 which would support its purported role in endosomal trafficking, 11, 12 there have been numerous studies implicating LITAF, which is highly expressed in monocytes, macrophages, lymph nodes, and spleen, 53 as an important mediator in systemic and chronic inflammation. 54 Earlier studies identified LITAF as a lipopolysaccharide-induced transcription factor for TNF (tumor necrosis factor)-α. 55 Despite some controversy regarding these findings, 14, 21 recent studies using highly specific chromatin immunoprecipitation, confirmed LITAF’s role as a transcription factor. For example, lipopolysaccharide-induced LITAF acts as a transcriptional activator for TNF-α, which mediates a proinflammatory and profibrogenic pattern in nonalcoholic fatty liver disease. 23 We, therefore, entertained the possibility of LITAF-dependent transcriptional effects on Cav1c expression. However, our quantitative polymerase chain reaction data clearly indicated that Cav1c transcript levels were not changed on LITAF overexpression in neonatal cardiomyocytes (Figure IVC in the Data Supplement), despite significantly decreased Cav1c protein levels (Figure IVA in the Data Supplement). Currently, we cannot rule out any possible LITAF-mediated transcriptional effects on other ion channels or molecules underlying AP formation. Of note, we have not observed any significant changes in the total protein levels of HERG, KvLQT1, NCX, SERCA2, and PLN (phos-
LITAF, a Potential Candidate to Alter QT Interval

Based on the effects we have observed of LITAF on calcium transients in zebrafish (Figure 1), which largely depend on Ca\(^{2+}\) influx through transmembrane Ca\(^{2+}\) channels, and \(I_{\text{Ca,L}}\) in rabbit cardiomyocytes (Figure 3), we propose to add genetic variants of LITAF to the growing list of genetic risk factors for sporadic or drug-induced arrhythmias. Identification and expansion of molecular risk factors could help to identify vulnerable patients and aid in the development of new strategies targeting individuals with a prolonged QT interval. The high degree of sequence conservation among known LITAF orthologs implies a conservation of function during vertebrate evolution from bony fish to mammals. Here, we provide strong evidence for a role of LITAF in regulation of calcium transients in zebrafish embryos (Figure 1) and in the control of LTCC levels on the membrane of rabbit cardiomyocytes (Figures 2 and 3). The association between LITAF and QT interval variation has been reported in various genome-wide association studies. Indeed, computer simulation of rabbit ventricular myocytes predicted a modest APD shortening in the presence of overexpressed LITAF, primarily because of the compensatory response of NCX. We observed similar magnitude of shortening of the APD in 3wRbCM that did not reach statistical significance in the setting of large variance in the APD of cultured myocytes possibly due to cell-to-cell variability in remodeling of ion channels in culture. Complementing these experiments, LITAF knockdown in zebrafish resulted in prolongation APD that did not reach statistical significance. These observations may reflect a role for LITAF in damping dynamic changes in excitation similar to those reported for other regulators of membrane protein turnover. In this framework, changes in LITAF activity in either direction create vulnerabilities to other modulators of APD, whether genetic or environmental.

Conclusions

Here, we provide both in vivo and in vitro data that imply a role for LITAF in the regulation of LTCC in the heart through modulation of the activity of the HECT ubiquitin ligase NEDD4-1. The end result is ubiquitination and subsequent lysosomal degradation of Cav\(_{\alpha 1c}\) (Figure VI in the Data Supplement). This, in turn, results in lower LTCC levels on the cell surface and decreased \(I_{\text{Ca,L}}\) of cardiomyocytes. We conclude that LITAF controls membrane levels and function of LTCC and is a novel regulator of cardiac excitation.
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