The endosomal trafficking regulator LITAF controls the cardiac Nav1.5 channel via the ubiquitin ligase NEDD4-2

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The QT interval is a recording of cardiac electrical activity. Previous genome-wide association studies identified genetic variants that modify the QT interval upstream of LITAF (lipopolysaccharide-induced tumor necrosis factor-α factor), a protein encoding a regulator of endosomal trafficking. However, it was not clear how LITAF might impact cardiac excitation. We investigated the effect of LITAF on the voltage-gated sodium channel Nav1.5, which is critical for cardiac depolarization. We show that overexpressed LITAF resulted in a significant increase in the density of Nav1.5-generated voltage-gated sodium current \( I_{\text{Na}} \) and Nav1.5 surface protein levels in rabbit cardiomyocytes and in HEK cells stably expressing Nav1.5. Proximity ligation assays showed co-localization of endogenous LITAF and Nav1.5 in cardiomyocytes, whereas co-immunoprecipitations confirmed they are in the same complex when overexpressed in HEK cells. In vitro data suggest that LITAF interacts with the ubiquitin ligase NEDD4-2, a regulator of Nav1.5. LITAF overexpression downregulated NEDD4-2 in cardiomyocytes and HEK cells. In HEK cells, LITAF increased ubiquitination and proteasomal degradation of co-expressed NEDD4-2 and significantly blunted the negative effect of NEDD4-2 on \( I_{\text{Na}} \). We conclude that LITAF controls cardiac excitability by promoting degradation of NEDD4-2, which is essential for removal of surface Nav1.5. LITAF-knockout zebrafish showed increased variation in and a nonsignificant 15% prolongation of action potential duration. Computer simulations using a rabbit-cardiomyocyte model demonstrated that changes in \( \text{Ca}^{2+} \) and \( \text{Na}^{+} \) homeostasis are responsible for the surprisingly modest action potential duration shortening. These computational data thus corroborate findings from several genome-wide association studies that associated LITAF with QT interval variation.

The voltage-gated sodium channel Nav1.5 is responsible for the initial upstroke of cardiac action potential (1, 2). Post-translational modifications such as phosphorylation or ubiquitination are essential for correct expression and function of Nav1.5 (1). The activity and density of Nav1.5 channels at the membrane depend on forward trafficking, stability, and domain-targeting mediated by anchoring proteins and retrograde trafficking (3). Retrograde trafficking of cardiac Nav1.5 depends on the E3 ubiquitin ligase NEDD4-2 (neural precursor cell-expressed developmentally down-regulated 4 type 2), which accelerates the degradation of Nav1.5 by ubiquitination (4, 5). NEDD4-2 is highly expressed in the heart (6), where it down-regulates Nav1.5 through PXY motif recognition by its WW domains (5). However, the details of this regulation are not well-understood.

Genetic modifications in the SCNSA gene, which encodes Nav1.5, cause inherited long QT syndrome 3, Brugada syndrome, atrial fibrillation, sick sinus syndrome, progressive cardiac conduction defect, or dilated cardiomyopathy (reviewed by Li et al. (7)). Also, dysfunction of Nav1.5 in myocardial ischemia and heart failure is proarrhythmic (2, 8–10). Several genome-wide association studies for loci that modify the QT interval and the risk of sudden cardiac death (11–13) have identified three SNPs located in or near genes encoding proteins involved in ubiquitination (RNF207, RFFL, and LITAF) (14, 15). LITAF (lipopolysaccharide-induced tumor necrosis factor-alpha factor) is a regulator of endosomal trafficking (16–18) and inflammatory cytokines (19–21) and an adapter molecule for members of the NEDD4 (neural precursor cell-expressed developmentally down-regulated protein 4)–like family of E3 ubiquitin ligases (17, 22). The N terminus of LITAF contains two PXY motifs, which are essential for interacting with members of the NEDD4 family of HECT (homologous to the E6-AP C terminus) domain ubiquitin ligases via their WW domains (17, 22, 23). LITAF also interacts with members of the ESCRT (endosomal sorting complex required for transport) family, including TSG101 (tumor susceptibility gene 101) and STAM1 (signal-transducing adaptor molecule 1), recruiting them to the early endosomal membrane and controlling endosome-to-lysosome trafficking and exosome formation (16–18). Mutations clustered around the hydrophobic region required for membrane localization in LITAF cause Charcot–Marie–Tooth disease, an inherited peripheral neuropathy. They also result in mislocalization and impaired endosome-to-lysosome trafficking of membrane proteins (16, 24). Importantly, the genetic variant rs8049607 located within an intergenic enhancer region (25) is associated with a very modest QT interval prolongation.
of 1.2 ms (11–13). This variant (rs8049607) is associated with reduced LITAF mRNA transcript levels in the left ventricle (26, 27). Thus, a reduction in LITAF prolongs the QT interval.

Based on the genome-wide association studies’ findings (11–13) 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. Indeed, we have recently shown that LITAF acts as an adaptor protein promoting NEDD4-1–mediated ubiquitination and subsequent degradation of L-type calcium channels (LTCCs), and gain of function of LITAF is associated with shortening of action potential duration (APD) (27). In this study, we present data that support an additional role for LITAF in modulating membrane density and function of cardiac Nav1.5 via the ubiquitin ligase NEDD4-2. Uniquely, gain of function of LITAF increases the expression of sodium channels in the membrane.

**Results**

**The voltage-gated sodium current $I_{Na}$ is regulated by LITAF in 3-week-old rabbit cardiomyocytes**

To investigate any possible effect of LITAF on the Nav1.5 channel and its generated voltage-gated sodium current $I_{Na}$, we used cultured 3-week-old rabbit cardiomyocytes (3wRbCM). We developed and used this model to study various ion channels underlying action potential duration (27, 28). For example, 3-week-old rabbit cardiomyocytes cultured for 48 h display a stable $I_{Na}$ current (Fig. 1A). The cells were transduced with adenovirus encoding GFP and hemagglutinin (HA)–tagged LITAF. Overexpression of LITAF caused a significant increase (27.4%) in peak $I_{Na}$ density (from $-19.3 \pm 2.2$ pA/pF to $-24.6 \pm 2.21$ pA/pF; $p = 0.0073$; Fig. 1B), yet there were no changes in voltage-dependent activation and inactivation kinetics (Fig. 1C). Western blotting results show that total Nav1.5 protein levels were significantly up-regulated (76.7%) in LITAF-overexpressing 3wRbCM ($p < 0.05$; Fig. 1D).

**LITAF controls Nav1.5 channel expression on the cell surface in HEK cells**

Next, we switched to HEK cells as they are frequently used to study Nav1.5 in vitro (5, 29, 30). We used HEK cells that stably co-express Nav1.5 and GFP, to confirm our data obtained with 3wRbCM. HEK cells were co-transfected with expression plasmids for LITAF and red fluorescent protein (DsRed) or GFP and DsRed (control). Patch clamp results show that LITAF increased (20.1%) Nav1.5 current density from $-80.6 \pm 12.9$ pA/pF to $-96.8 \pm 13.5$ pA/pF; $p < 0.05$; Fig. 2B). Importantly, a significant concomitant increase in total Nav1.5 channel expression was noted (Fig. 2C). Surface biotinylation experiments were carried out to confirm that membrane expression of Nav1.5 was also significantly elevated upon LITAF overexpression (Fig. 2D), which is consistent with higher $I_{Na}$ peak density in the presence of exogenous LITAF (Fig. 2C).

**Cardiac LITAF regulates Nav1.5 expression**

**Close-proximity interaction between LITAF and Nav1.5 channels**

Because our data suggested a functional interaction between LITAF and Nav1.5, we performed co-immunoprecipitation experiments on HEK cells stably expressing V5-tagged Nav1.5. The cells were co-transfected with plasmid encoding HA-tagged LITAF or empty control vector. Cell extracts were incubated with V5 antiserum, immunoprecipitated, separated by SDS-PAGE, transferred to membrane, and probed with anti-Nav1.5 antibody. Western blotting analyses suggest that LITAF is found in a protein complex with Nav1.5 (Fig. 3A). Additionally, we performed in situ PLA to look for any co-localization between these two molecules in 3wRbCM (Fig. 3B). The specificity of the assay was shown by the lack of staining using mouse anti-LITAF or rabbit anti-Nav1.5 as negative controls. The appearance of puncta with the combination of mouse anti-LITAF and rabbit anti-Nav1.5 supports proximity between LITAF and Nav1.5 in cardiomyocytes.

**LITAF blunts the NEDD4-2–dependent down-regulation of $I_{Na}$ in HEK cells**

NEDD4-2–dependent ubiquitination is a prerequisite for the degradation of surface Nav1.5 (4, 5). Because we have previously established physical and functional interactions between LITAF and the ubiquitin ligase NEDD4-1 (27), we entertained the possibility LITAF may also regulate NEDD4-2 with respect to Nav1.5. Therefore, we first looked for any physical interaction between NEDD4-2 and LITAF, which could be mediated by four WW domains of NEDD4-2 and two PXX motifs of LITAF (17, 31). Total lysates of stable HEK cells transiently expressing HA-tagged LITAF and FLAG-tagged NEDD4-2 were immunoprecipitated with FLAG antibody. The resulting immunoprecipitates were subjected to Western blotting. Fig. 4A shows co-precipitated LITAF indicating that LITAF and NEDD4-2 are found in the same protein complex. We also noticed that LITAF significantly reduced levels of co-expressed NEDD4-2 by 39% (Fig. 4A). Next, we wanted to assess the possible role of LITAF and NEDD4-2 in the homeostasis of Nav1.5. To this end, we transiently transfected HEK cells stably expressing Nav1.5 and GFP. Not surprisingly, co-expressed NEDD4-2 significantly decreased peak $I_{Na}$ density (e.g. by 68%, viz. from $-71.1 \pm 12.7$ pA/pF to $-22.5 \pm 3.8$ pA/pF; $-10$ mV; $p < 0.01$; Fig. 4B), which is in agreement with a previous study by van Bemmelen et al. (5). Co-expressed LITAF, however, partially reversed the negative effect of NEDD4-2 on $I_{Na}$ density (from $-22.5 \pm 3.8$ pA/pF to $-37.6 \pm 13.0$ pA/pF; $-10$ mV; $p < 0.01$; Fig. 4B). Thus, this 52% recovery of $I_{Na}$ in the presence of co-expressed LITAF is in line with the aforementioned 39% LITAF-dependent drop in NEDD4-2 levels. In summary, these functional data corroborate a role for LITAF modulating membrane expression of Nav1.5 by regulating NEDD4-2 ubiquitination-mediated degradation.
Overexpression of LITAF results in ubiquitination and proteasomal degradation of NEDD4-2 in HEK cells

Because the data presented in Fig. 4 suggest a functional interaction between LITAF and NEDD4-2 regulating Nav1.5 expression on the membrane, we wanted to investigate the possible role of LITAF in the regulation of NEDD4-2. To this end, we measured endogenous NEDD4-2 levels in 3-week-old and neonatal rabbit cardiomyocytes (NRbCM) overexpressing LITAF. We noted that LITAF overexpression reduced total levels of NEDD4-2 by 30% (3wRbCM) and 50% (NRbCM), respectively (Fig. 5, A and B). Similarly, LITAF overexpression down-regulated endogenous NEDD4-2 levels by 60% in HEK cells (Fig. 5, C and E). Lastly, co-expression of LITAF and FLAG-tagged NEDD4-2 in HEK cells resulted in an ~90% down-regulation of NEDD4-2–FLAG (Fig. 5, D and E).

We reasoned that LITAF overexpression likely caused ubiquitin-mediated degradation of NEDD4-2 in the various cell types tested. To test this hypothesis, we co-transfected expression plasmids for HA-tagged ubiquitin, FLAG-tagged NEDD4-2, FLAG-tagged LITAF, or control plasmid into HEK cells. Total cell extracts prepared 2 days later were immunoprecipitated with an anti-HA antibody to enrich for HA-ubiquitinated protein. The ubiquitinated protein fraction was separated by size using SDS-PAGE, transferred to a membrane, and probed against NEDD4-2. Western blotting data depicted in Fig. 6A indicate a significant, severalfold, LITAF-dependent increase in a single NEDD4-2 band implying likely monoubiquitination of the ubiquitin ligase. Next, we tested the pathways potentially responsible for LITAF-dependent NEDD4-2 degradation. We treated HEK cells expressing NEDD4-2, LITAF, or control plasmid with the selective proteasome inhibitor MG132 (32) or the lysosomal inhibitor chloroquine (33) for 24 h. Treatment of cells with MG132, but not treatment with chloroquine, partially prevented the LITAF-mediated down-regulation of NEDD4-2 (Fig. 6B). In summary, our data suggest that LITAF overexpression causes NEDD4-2 monoubiquitination and its subsequent degradation, in part through proteasomes. This, in turn, could account for the LITAF-mediated up-regulation of Nav1.5 expression and I\textsubscript{Na} in cardiomyocytes.

Computer modeling of LITAF overexpression

Several genome-wide association studies (11–13) have implied a role for LITAF in QT interval and, therefore, in action potential regulation. Hence, we were interested in whether the LITAF-dependent effects on I\textsubscript{Na} (in this study) and L-type calcium current I\textsubscript{Ca,L} (27) could account for any changes in APD. To this end, we used a physiologically detailed model of rabbit ventricular myocyte with membrane voltage coupled to spatially distributed subcellular calcium dynamics. This model is improved from Moshal et al. (27) through several modifications.
First, we included the late sodium current \( (I_{NaL}) \) from Hwang et al. (34). Second, based on the voltage-clamp experiments, we modeled the effects of LITAF overexpression by increasing the conductance of both \( I_{Na} \) and \( I_{NaL} \) by 35% and reducing the number of LTCCs by 50% as in our previous study (27). We modeled the myocyte in current-clamp mode at 2.5 Hz (400-ms pacing cyclic length) and recorded the transmembrane voltage \( (V) \), the calcium transient, and several sarcolemmal currents after the intracellular sodium concentration \( [Na^+]_i \) reached steady state. We investigated three different conditions: control GFP with intracellular sodium concentration \( [Na^+]_i \) unclamped, LITAF overexpression with \( [Na^+]_i \), unclamped, and LITAF overexpression with \( [Na^+]_i \), clamped at a value of 11 mM corresponding to the average steady-state \( [Na^+]_i \) value under GFP; the latter condition was studied to dissect the effect of a change of steady-state \( [Na^+]_i \) on action potential duration. The comparison of GFP and LITAF simulations with unclamped \( [Na^+]_i \), (red and blue traces in Fig. 7) demonstrate that the 50% decrease of LTCC number (Fig. 7D) together with the 35% increase of sodium channel conductance \( (I_{Na} \ and \ I_{NaL}) \) in Fig. 7, I and J) shortens the APD from 218 ms with GFP to 196 ms with LITAF (Fig. 7A). The results further show that this modest change of APD can be attributed to the subtle knock-on effect of \( I_{Ca,L} \) on other currents mediated by the changes in \( [Ca^{2+}]_i \) transient and \( [Na^+]_i \), preliminarily explored in Moshal et al. (27). More specifically, the decrease in the \( [Ca^{2+}]_i \) transient amplitude (Fig. 7B) caused by the reduction of \( I_{Ca,L} \) (Fig. 7D) decreases the amplitude of both the forward and reverse modes of the \( Na^+/Ca^{2+} \) exchanger current \( (I_{NCX}) \), thereby causing a net decrease in \( [Na^+]_i \), caused by reduction of the net forward mode of \( I_{NCX} \) averaged over one pacing cyclic length. This decrease in \( [Na^+]_i \) in turn decreases the repolarizing Na,K-ATPase current \( (I_{NaK}) \) (Fig. 7F), thereby partially counter-balancing the effect of \( I_{Ca,L} \) reduction on APD shortening. The results also show that although the magnitude of the rapid component of the delayed rectifier potassium current \( (I_{Kr}) \) remains largely unchanged (Fig. 7H), the lowered action potential plateau

**Figure 2.** LITAF increases \( I_{Na} \) as well as surface expression of Nav1.5 in stable HEK cells. The cells stably co-expressing Nav1.5 and GFP were co-transfected with LITAF- and DsRed-expressing plasmids or control (GFP and DsRed). A, typical \( I_{Na} \) traces in GFP- and LITAF-expressing HEK cells. B, current-voltage relationship for \( I_{Na} \). \( I_{Na} \) was activated from \(-100 \) mV holding potential by depolarizing steps up to +40 mV in 10-mV increments. C, top panel, total expression of Nav1.5 and LITAF-HA. Bottom panel, respective change in Nav1.5 expression, normalized to tubulin (all values are means ± S.E.; Student’s t test; n = 10). The uncropped probed membrane is shown in Fig. S1. D, total and surface protein expression of Nav1.5 and LITAF. Stable HEK cells were transfected with LITAF or GFP (control) expression plasmids. Cell-surface protein was biotinylated using sulfo-NHS-S-biotin, purified with NeutrAvidin beads from total cell lysates, subjected to SDS-PAGE, and blotted onto a polyvinylidene difluoride membrane. A representative immunoblot shows cell-surface and total lysate expression of Nav1.5, LITAF-HA, and tubulin (top panel) (the asterisk indicates an unspecific band). Respective changes in Nav1.5 expression, normalized to tubulin. All values are means ± S.E. (n = 5) (bottom).
Figure 3. Physical interaction between LITAF and Nav1.5 in HEK cells and 3wRbCM. A, V5 IP of lysates from HEK cells stably expressing V5-tagged Nav1.5 and transfected with plasmids for HA-tagged LITAF or empty expression plasmid (control) (n = 3). The right panel shows immunoprecipitated Nav1.5 and co-precipitated LITAF-HA and thus an interaction between LITAF and Nav1.5 (the asterisk indicates the light chain of the IP capture antibody). Input levels of Nav1.5 and LITAF-HA are shown in the left panel. B, Duolink in situ proximity ligation assay using mouse anti-LITAF and rabbit anti-Nav1.5 antibodies in 3wRbCM. The co-localization between molecules is indicated by red puncta (left panel). Virtually no puncta were detected in negative controls in which only one antibody was used, i.e. rabbit anti-Nav1.5 (middle panel) or mouse anti-LITAF antibodies (right panel). The nuclei were stained with DAPI (blue). Depicted merged confocal images (bright field, DAPI, and Texas Red) are representative of each condition. Scale bar, 50 μm.

Figure 4. LITAF partially abolishes NEDD4-2–dependent down-regulation of I_{Na} in HEK cells. A, co-immunoprecipitation of cell lysates from HEK cells transfected with plasmids for NEDD4-2–FLAG and LITAF-HA or just NEDD4-2–FLAG for 48h (n = 3). Top panel, the top part shows an interaction between immunoprecipitated NEDD4-2–FLAG and co-precipitated LITAF-HA (the asterisk indicates the light chain of the IP capture antibody), whereas the bottom part displays input levels of NEDD4-2–FLAG and LITAF-HA. Bottom panel, respective changes in input NEDD4-2 expression, normalized to tubulin. All values are means ± S.E. (n = 3). B, current–voltage relationships of I_{Na} peak currents for baseline conditions from cells expressing. GFP (control), NEDD4-2, or NEDD4-2 and LITAF (means ± S.E.). Two-way analysis of variance for repeated measures revealed significant differences in I_{Na} between all groups: GFP (control) versus NEDD4-2: F = 80.05, p < 0.0001. NEDD4-2 versus NEDD4-2 + LITAF: F = 11.42, p = 0.0008. GFP versus NEDD4-2 + LITAF: F = 15.28, p = 0.0001.
significantly suppresses the fast component of the delayed rectifier potassium current (\(I_{KS}\)) (Fig. 7G), despite a modest APD shortening, thereby further contributing to counterbalancing APD shortening. Although both hyperpolarizing \(I_{NaK}\) and \(I_{KS}\) are reduced under LITAF compared with GFP, the simulation results with \([Na^+]_i\) clamped at the higher 11 mM steady-state value corresponding to GFP (green traces in Fig. 7) clearly show that the reduction of steady-state \([Na^+]_i\) is the main causal mechanism underlying the smaller than expected APD shortening in the presence of significant \(I_{Ca,L}\) reduction. In particular, without this decrease, when \([Na^+]_i\) is clamped at its higher GFP value, \(I_{NaK}\) is only slightly lower under LITAF than GFP (red and blue traces in Fig. 7), thereby yielding a more significant APD shortening (Fig. 7A). Importantly, APD is significantly shortened despite further reduction of \(I_{KS}\) as a direct effect of membrane voltage (Fig. 7G), and peak \(I_{NaL}\) is only slightly reduced. An additional simulation carried out with unclamped \([Na^+]_i\) and modeling only the effect of LITAF overexpression on \(I_{Ca,L}\) but not that on \(I_{Na}\) and \(I_{NaL}\), confirmed that \(I_{Na}\) and \(I_{NaL}\) increase has a minor effect on both APD and \([Na^+]_i\) (results not shown). This is because, with LITAF overexpression, the reductions in \(I_{NaK}\) and \(I_{KS}\) contribute more than the modest increase in \(I_{NaL}\) to counterbalancing the decrease of APD. Moreover, the fast component of \(I_{Na}\) makes a negligible contribution to \([Na^+]_i\), because of its short ~1-ms duration (red and blue traces in Fig. 7), and the modest increase of \(I_{NaL}\) (Fig. 7J) is insufficient to counterbalance the decrease of \([Na^+]_i\) caused by reduced \(I_{NCX}\) with LITAF overexpression. In summary, the computer modeling results demonstrate that changes in \(Ca^{2+}\) and \(Na^{+}\) homeostasis are primarily responsible for the more modest than expected APD shortening in the presence of LITAF overexpression. The decrease of functional \(I_{Ca,L}\) current density causes a decrease of \(Ca^{2+}\) transient amplitude that in turn decreases \(I_{NCX}\) and thus \([Na^+]_i\) and \(I_{NaL}\). The decrease in \(I_{Ca,L}\) lowers the action potential plateau that in turn reduces the \(I_{KS}\). Reduction of hyperpolarizing \(I_{NaK}\) and \(I_{KS}\) then jointly contribute to counterbalancing the APD shortening because of decreased \(I_{Ca,L}\) with sodium current enhancement having a relatively small effect.

To investigate the role for LITAF on APD in vivo, we created a LITAF KO zebrafish line (c.45_55del), using Crispr/Cas9.
Compared with WT fish, we observed a nonsignificant modest 14.4% increase in APD in homozygous KO zebrafish (mean ventricular APD80 ± S.E.: homozygous 301 ± 18.7 ms (n = 21) versus WT 263 ± 13 ms (n = 13); p = 0.16). Notably, there is a significant increase in the variations of the APD of the KO zebrafish as compared with WT fish (p < 0.05). Of note, we previously observed insignificant shortening of APD in 3-week-old rabbit cardiomyocytes overexpressing LITAF in agreement with computer modeling results (data not shown), whereas morpholino-mediated down-regulation of LITAF in zebrafish embryos resulted in prolongation of APD that did not reach statistical significance (27). This suggests that decreased [Na+]i and I\textsubscript{NaK} as a knock-on effect of decreased I\textsubscript{Ca,L} current density may be a common mechanism for the modest change of APD in both rabbit and zebrafish cardiomyocytes. Even though I\textsubscript{Ks} assists I\textsubscript{NaK} in counterbalancing the effect of decreased I\textsubscript{Ca,L} on APD, I\textsubscript{Ks} is not significant enough to compensate for the decrease in I\textsubscript{Ca,L} current density.
APD in rabbit cardiomyocytes, decreased [Na\(^+\)], and \(I_{\text{NaK}}\) still play a dominant role in keeping APD almost constant. Hence, even though \(I_{\text{Na}}\) is not significantly expressed in zebrafish (35), decreased [Na\(^+\)], and \(I_{\text{NaK}}\) may suffice to counterbalance the effect of LITAF on \(I_{\text{CaL}}\) current density in zebrafish.

**Discussion**

Previously, several genome-wide association studies have identified SNPs located upstream of the LITAF gene, encoding a regulator of endosomal trafficking (16, 17, 36), associated with QT interval (11–13). In the present study, we provide evidence that LITAF controls the voltage-gated sodium current \(I_{\text{Na}}\) in rabbit cardiomyocytes. The main finding of this study is that LITAF increases \(I_{\text{Na}}\) and expression of Nav1.5 channel on the membrane by promoting ubiquitination and degradation of the ubiquitin ligase NEDD4-2, which is indispensable for Nav1.5 turnover (4, 5). The voltage-gated sodium channel Nav1.5 is critical for the generation and conduction of cardiac action potentials. Mutations and changes in the expression level of Nav1.5 are associated with cardiac arrhythmias and sudden cardiac death as reviewed by Song *et al.* (37).

Because forward and retrograde trafficking of ion channels affect their overall function under physiological as well as pathological conditions, a large number of studies identified molecular factors involved in Nav1.5 trafficking and degradation (reviewed by Rook *et al.* (1)). For example, van Bemmel *et al.* (5) showed that the HECT ubiquitin ligase NEDD4-2 could bind through its WW domains to the PXY motif found at the C terminus of Nav1.5. Overexpression of NEDD4-2, but not the closely related NEDD4-1, increased Nav1.5 ubiquitination and subsequent degradation in HEK cells. Follow-up studies identified the ubiquitin-activating enzymes UBE1 and UBA6 (38), as well as the ubiquitin-conjugating enzyme UBC9 (39), to be required for NEDD4-2–dependent ubiquitination of Nav1.5 in neonatal rat cardiomyocytes and HEK cells. Our data suggest that this NEDD4-2–dependent control of Nav1.5 turnover is regulated by LITAF, previously identified as a protein involved in endosomal trafficking and inflammation (16–21).

We provide evidence that 1) LITAF is found in protein complexes with Nav1.5 and NEDD4-2 in HEK cells and in 3wRbCM; 2) LITAF overexpression resulted in higher \(I_{\text{Na}}\) and total \(I_{\text{Na}}\); and 3) LITAF co-expression blunted the negative effect of NEDD4-2 on \(I_{\text{Na}}\) and 4) LITAF lowered exogenous and endogenous NEDD4-2 levels by promoting its ubiquitination and degradation. Thus, it is possible that LITAF and the small NEDD4 family-interacting proteins (NDFIPs), NDFIP1 and NDFIP2 (40) may share common mechanisms in regulating members of the NEDD4 family.

Interestingly, we have recently published that LITAF is also a regulator of L-type calcium channels in zebrafish and rabbit cardiomyocytes (27). Here, LITAF acts as an adaptor protein and activator of the HECT ubiquitin ligase NEDD4-1. This led to subsequent degradation of L-type calcium channel, thereby controlling its membrane levels, \(I_{\text{Ca,L}}\), and thus cardiac excitation. Currently, we can only speculate as to why LITAF regulates NEDD4-1 and NEDD4-2 differently with respect to their target molecules LTCC and Nav1.5 in cardiomyocytes. Similar to NDFIPs, we noted that LITAF overexpression lowered the amount of both NEDD4-2 (Fig. 5) and NEDD4-1,5 which was accompanied by increased ubiquitination (Fig. 6A). Importantly, LITAF also enhanced NEDD4-1-dependent ubiquitination of LTCC (27). It is conceivable that LITAF may act similarly to NDFIPs (40), possibly redirecting NEDD4-2 from the surface, where it would ubiquitinate Nav1.5, to another cellular location for sequestration or to target other substrates. Nevertheless, the net effect is to set a ratiometric relationship between LTCC and Nav1.5 at the membrane, thus regulating repolarization and its dynamics.

Although we have recapitulated LITAF’s positive effect on \(I_{\text{Na}}\) in stable HEK cells (Fig. 2, A and B) and provided a molecular explanation for this phenomenon, viz. down-regulation of NEDD4-2 required for Nav1.5 degradation (Fig. 5), other mechanisms may contribute to the LITAF-dependent increase in \(I_{\text{Na}}\) in cardiomyocytes. For example, Luo *et al.* (41) have shown that elevated intracellular calcium levels increase NEDD4-2 mRNA expression in neonatal rat cardiomyocytes. Higher NEDD4-2 protein levels, in turn, reduced \(I_{\text{Na}}\) and \(I_{\text{NaK}}\). Because LITAF overexpression in rabbit cardiomyocytes resulted in lower \(I_{\text{CaL}}\) and calcium transients (27) but increased \(I_{\text{Na}}\) (Fig. 1), it is possible that resulting lower intracellular calcium levels could reduce NEDD4-2 mRNA expression, diminishing the negative impact NEDD4-2 has on surface \(I_{\text{Na}}\) protein levels and \(I_{\text{NaK}}\). Several studies reported lower Nav1.5 protein levels and \(I_{\text{Na}}\) in ischemia and heart failure (42, 43), likely increasing the risk for arrhythmias. In ischemia, no changes in Nav1.5 transcript levels were detected, implying accelerated degradation of Nav1.5 protein. At least one ubiquitin-dependent mechanism may account for the decrease in Nav1.5 levels in cardiac disease. Increased calcium concentrations in the cytosol caused by malfunctioning RYR2 increased NEDD4-2 expression lowering Nav1.5 levels, as data from volume-overload heart failure rat hearts suggested (41).

In agreement with the experiments associated with LITAF overexpression in 3-week-old rabbit cardiomyocytes and with LITAF KO in zebrafish (27), the computational simulation shows that LITAF overexpression shortens APD (Fig. 7). This effect takes place via the decrease in \(I_{\text{Ca,L}}\) resulting from the activation of HECT ubiquitin ligase NEDD4-1 (27). The consequence of decreased \(I_{\text{Ca,L}}\) is to reduce the Ca\(^{2+}\) transient amplitude that in turn reduces [Na\(^+\)]. Together with the lowered APD plateau caused by decreased \(I_{\text{Ca,L}}\), the decrease in [Na\(^+\)] reduces \(I_{\text{NaK}}\). Another effect of the lowered APD plateau is the decrease of \(I_{\text{Ks}}\). Reduction of hyperpolarizing \(I_{\text{NaK}}\) and \(I_{\text{Ks}}\) then jointly contribute to counterbalancing the APD shortening. The simulation also shows that the increase in sodium currents \(I_{\text{Na}}\) and \(I_{\text{NaK}}\) caused by LITAF overexpression has a minor effect on [Na\(^+\)], and APD. The overall effect of LITAF overexpression on \(I_{\text{Ks}}\) and \(I_{\text{Ca,L}}\) would lead to shorter APD and QT interval.

In summary, we conclude that LITAF is a novel regulator of Nav1.5 and \(I_{\text{NaK}}\). The data provided in this study and the

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5 N. N. Turan, K. S. Moshal, K. Roder, B. C. Baggett, A. Y. Kabakov, S. Dhakal, R. Teramoto, D. Y. E. Chiang, M. Zhong, A. Xie, Y. Lu, S. C. Dudley, Jr., C. A. MacRae, A. Karma, and G. Koren, unpublished data.
Evidence of its complex role in regulating multiple ion currents make LITAF an interesting target for innovative strategies in the prevention and treatment of ventricular arrhythmias, not least those associated with reduced Nav1.5 function and $I_{Na}$.

### Experimental procedures

#### DNA

An expression cassette consisting of the cardiac CMV-enhanced 0.26-kb rat myosin late chain promoter (44), human β-globin intron, a fusion between the human LITAF ORF and three hemagglutinin (HA) tags, an internal ribosome entry site, the humanized Renilla reniformis GFP (hrGFP) ORF, and the human growth hormone polyadenylation signal (hGH1polyA) was cloned into pENTR 1A Dual (Thermo Fisher). The expression cassette was then transferred to the vector pAd/PL-DEST (Thermo Fisher) using the Gateway cloning system (Thermo Fisher). 293A cells (Thermo Fisher) were transfected with Paclidigested pAd/PL-DEST-CMV-MLC-LITAF-HA-hrGFP, and adenoviral stocks were prepared according to the manufacturer. As control, we created the vector pAd/PL-DEST-CMV-MLC-hrGFP that allows the expression of hrGFP in adenovirus. An expression cassette consisting of the CMV promoter, human β-globin intron, 3×HA LITAF, an internal ribosome entry site, hrGFP, and hGH1polyA was cloned into pENTR 1A Dual to obtain the mammalian expression vector pENTR-CMV-LITAF-HA-hrGFP. Similarly, the FLAG-tagged LITAF expression plasmid was created: pENTR-CMV-LITAF-FLAG-hrGFP. As control vector, pENTR-CMV-hrGFP—expressing hrGFP was created. The plasmid pcDNA3-Nedd4-2-FLAG was obtained from Dr. Sharad Kumar (Centre for Cancer Biology, University of South Australia and SA Pathology). pRK5-HA-ubiquitin-WT expressing HA-tagged ubiquitin (45) was purchased from Addgene (Addgene ID 17608). pDsRed-C1 vector was originally obtained from Clontech.

#### Transfections

HEK cells and HEK cells stably co-expressing Nav1.5 and GFP were cultured in DMEM (Thermo Fisher) supplemented with 10% FBS. Transient transfections of HEK cells were performed for 48 h using Lipofectamine 2000 (Thermo Fisher) and following the manufacturer’s instructions. Typically, we transfected 200 ng of pENTR-CMV-LITAF-HA-hrGFP (or pENTR-CMV-hrGFP), 200 ng of pcDNA3-Nedd4-2-FLAG, 200 ng of pcDNA3, and 1.8 μl of Lipofectamine 2000 per 12-well (Figs. 5, C and D, and 6B) or 500 ng of pRK5-HA-Ubiquitin-WT, 500 ng of pENTR-CMV-LITAF-FLAG-hrGFP (or pENTR-CMV-hrGFP), 500 ng of pcDNA3-Nedd4-2-FLAG, 1.5 μg of pcDNA3 (Invitrogen), and 7.2 μl of Lipofectamine 2000 per 6-cm dish (Fig. 6A). For transient transfections of HEK cells stably expressing Nav1.5, we generally used 1600 ng of pENTR-CMV-LITAF-HA-hrGFP (or pENTR-CMV-hrGFP), 1600 ng of pcDNA3-Nedd4-2-FLAG (or pcDNA3), 800 ng of pENTR-CMV-hrGFP, 270 ng of pDsRed-C1, and 10 μl of Lipofectamine 2000 (Figs. 2, B–D; 3A; and 4, A and B).

### Preparation of rabbit cardiomyocytes

All animal experiments and procedures were approved by the Rhode Island Hospital Institutional Animal Care and Use Committee (reference nos. 0188-14 and 5013-17). 3-week-old ventricular cardiomyocytes were isolated from the hearts of 3-week-old NZW rabbits (both sexes) with standard enzymatic techniques using a Langendorff system. NZW rabbits were administered pentobarbital sodium (65 mg/kg IP) and heparin (1,000 units/kg IP). The filtered cells were maintained in 45 mM KCl, 65 mM potassium glutamate, 3 mM MgSO₄, 15 mM KH₂PO₄, 16 mM tauroine, 10 mM HEPES, 0.5 mM EGTA, and 10 mM glucose (pH 7.3) for half an hour. In five subsequent steps, the Ca²⁺ concentration was increased to 1.8 mM. The cells were centrifuged, resuspended in DMEM supplemented with 7% FBS and antibiotics, plated on laminin-coated cover glasses or tissue culture dishes. After 2–3 h, the medium was replaced and adenovirus (50 MOI) added to the cells. The cells were maintained at 37 °C with 5% CO₂, and ∼48 h later, the cells were used for patch clamping and biochemistry. The isolation of neonatal rabbit cardiomyocytes was exactly performed as described.

### Electrophysiological recordings

3-week-old rabbit cardiomyocytes were transduced with adenovirus ∼48 h before $I_{Na}$ recording. The experiments were conducted at 34–36 °C with an Axopatch-200B amplifier, Digidata 1440A, and pClamp 10 software (Axon Instruments). The signal was acquired at 20 kHz and filtered at 10 kHz. The whole-cell configuration was obtained in standard Tyrode solution containing 140 mM NaCl, 5.4 mM KCl, 0.33 mM NaH₂PO₄, 1.8 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 5.5 mM D-glucose, and pH 7.4 was adjusted with NaOH. The pipette solution contained 80 mM CsCl, 80 mM Cs aspartate, 1 mM MgCl₂, 1 mM CaCl₂, 11 mM EGTA, 10 mM HEPES, 5 mM Na₃ATP, and pH 7.3 was adjusted with CsOH. The pipette resistance was 1–2 MΩ. After obtaining the whole-cell configuration and compensation of the capacitive artifact and access resistance by 80%, we replaced the Tyrode solution with low sodium solution containing 100 mM N-methyl-D-glucamine, 20 mM tetraethylammonium chloride, 10 mM NaCl, 5 mM CsCl, 2 mM CaCl₂, 1.2 mM MgCl₂, 10 mM HEPES, 5 mM D-glucose, 0.01 mM nifedipine, and pH 7.4 was adjusted with HCl. To obtain $V_I$ curves for $I_{Na}$, the cells were depolarized from −100 mV holding potential to +60 mV by 200-ms depolarizing steps in 10-mV increments. The $I_V$ curve in Fig. 1B was fitted with OriginPro 2019 Boltzmann $I_V$ function, and the obtained $E_{rev}$ values have been used to calculate conductance as $g = I_{Na}(V - E_{rev})$ at different potentials for the activation curve in Fig. 1C. To study inactivation of $I_{Na}$, the 200-ms depolarizing steps were followed by a 220-ms step to −20 mV. The $I_{Na}$ peak values were always measured relatively to the tail current at the end of the corresponding voltage pulse. To study effects of LITAF and NEDD4-2 on $I_{Na}$ in stable HEK cells, we used the same solutions and voltage protocols as described above for 3-week-old rabbit cardiomyocytes.
Cardiac LITAF regulates Nav1.5 expression

Based on Moshal et al. (27), we applied the modifications described by the following paragraphs. All the modified parameters are listed in Table 1.

Following Hwang et al. (34), we included the late sodium current (I_{NaL}). The model is described by the following set of equations.

\[ E_{Na} = \frac{RT}{F} \log \left( \frac{[Na^+]_o}{[Na^+]_i} \right) \] \hspace{1cm} (Eq. 1)

\[ m_{i\infty} = \frac{1}{1 + \exp \left( -\frac{V - 54.728}{24.327} \right)} \] \hspace{1cm} (Eq. 2)

\[ h_{i\infty} = \frac{1}{1 + \exp \left( -\frac{V - 9.2715}{19.913} \right)} \] \hspace{1cm} (Eq. 3)

\[ I_{NaL} = g_{NaL} m_{i\infty}^3 h_{i\infty}^2 (V - E_{Na}) \] \hspace{1cm} (Eq. 4)

Based on the voltage-clamp experiments, we modeled the effects of LITAF overexpression by increasing the conductance of both I_{Na} and I_{NaL} by 35% in addition to reducing the number of LTCCs by 50% as in the work of Moshal et al. (27).

Aquaculture

The experiments were performed on zebrafish (Danio rerio) on the AB/Tuebingen (AB/Tu) background in accordance with animal protocols approved by the Harvard Medical School Institutional Animal Care and Use Committee. Care and breeding of zebrafish were performed as described previously at 28.5 °C, and embryos were maintained in standard E3 medium (49).

Generation of LITAF knockout in zebrafish

WT AB/Tu zebrafish were crossed, and the resultant embryos were injected with a solution containing crRNA targeting LITAF (ATGGAGAACACGCCCCTGTTGGG), AltR® tracrRNA, and AltR® S.p. HiFi Cas9 Nuclease V3 (all from Integrated DNA Technologies), according to the manufacturer’s instructions. These F0 embryos were raised up and outcrossed individually to WT AB/Tu fish. Resultant F1 embryos were sequenced to identify clutches with frameshift mutations that are predicted to be loss-of-function mutations. Selected F1 clutches were raised, fin-clipped, and sequenced to confirm the mutation in each individual fish. Adult fish heterozygous for an 11-bp deletion in exon 2 of LITAF (c.45_55del), which is predicted to result in a severely truncated protein of 22 amino acids (p.L16PfsX8), were in-crossed. The resultant F2 embryos were WT, heterozygous, and homozygous for the 11-bp deletion in expected Mendelian ratios and were used for downstream experiments.

Optical mapping of isolated zebrafish hearts

Optical mapping and signal processing were performed as previously described (49). Briefly, the hearts were isolated from zebrafish embryos at 72 h postfertilization and stained with the transmembrane potential–sensitive dye in the FluoVolt™

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**Table 1**

| Parameter | Definition | Value |
|-----------|------------|-------|
| g_{NaL} | I_{NaL} conductance (GFP) | 0.01 |
| g_{NaL} | I_{NaL} conductance (LITAF) | 0.0135 |
| g_{NaL} | I_{NaL} conductance (GFP) | 12.0 |
| g_{NaL} | I_{NaL} conductance (LITAF) | 16.2 |

Immunoblot analysis

Surface biotinylations, co-IP, and immunoblots were exactly carried out as in previous studies (14, 15, 27).

**In situ proximity ligation assay**

For PLA, 3wRbCM were plated on 12-mm laminin-coated circular glass coverslips and cultured for 3–5 h in 24-well plates. The cells were fixed with 4% paraformaldehyde at room temperature for 15 min followed by permeabilization at room temperature with 0.1% Triton X-100 for 10 min prior to the assay. PLA was performed according to the manufacturer’s instructions (Sigma: Duolink® in situ Red starter kit mouse/rabbit). After five washes with PBS, the cells were blocked for 30 min at 37 °C. Antibodies used for PLA were as follows: rabbit anti-Nav1.5 (Alomone; ASC-005; 1:100) and mouse anti-LITAF (Abnova; H00009516-B01P; 1:100). The images were captured using a Nikon A1R laser scanning confocal microscope, DAPI (excitation, 359; emission, 461), Texas Red (excitation, 595; emission, 612) filters for detection purposes at 60× zoom, and Elements software (Nikon).

**Rabbit ventricular myocyte model**

To study the effect of LITAF in myocytes, we used a physiologically detailed rabbit ventricular myocyte model of Moshal et al. (27). This model is based on earlier models developed by Restrepo et al. (46) and further improved by both Terentyev et al. (47) and Zhong et al. (48). Combining together a large number of ~16,000 diffusively coupled Ca²⁺ release units, this multiscale model successfully links the whole-cell level Ca²⁺ dynamics to the local subcellular Ca²⁺ dynamics in each Ca²⁺ release unit, which incorporates four LTCC and 100 ryanodine receptors, both implemented by Markov models. By including the Ca²⁺-dependent channels LTCC and Na⁺·Ca²⁺ exchanger and other sarcolemmal currents, this detailed model describes the bidirectional coupling of Ca²⁺·V_{m} dynamics, which is essential in cardiac electrophysiological behavior. The details of LTCC model and ryanodine receptor model are shown by Zhong et al. (48).

We carried out the current clamp simulations by pacing the myocytes at 2.5 Hz (400 ms) for three different conditions: control GFP with Na⁺], unclamped, LITAF overexpression with [Na⁺], unclamped, and LITAF overexpression with [Na⁺], clamped at a value of 11 mM corresponding to the average steady-state [Na⁺] value under GFP. For the [Na⁺], unclamped simulations, we collected the data after the 7 intracellular sodium concentration reached the steady state.
Cardiac LITAF regulates Nav1.5 expression

membrane potential kit (Thermo Fisher). The resultant fluorescence intensities were recorded with a high-speed CCD camera (RedShirtImaging), and images were analyzed using custom scripts in MATLAB.

Statistical analysis and curve fitting

Statistical analysis and curve fitting were performed with GraphPad Prism 8 and OriginPro 2019. The data are presented as means ± S.E. A difference was considered significant at p < 0.05.

Replicates

Throughout the study, we used biological replicates, i.e. different animals or different frozen HEK cell stocks (as indicated by N), and technical replicates (as indicated by n).

Data availability

All of the data are contained within the article.

Acknowledgments—We are indebted to Dr. Sharad Kumar (Centre for Cancer Biology, University of South Australia and SA Pathology) for providing pcDNA3–Nedd4–2–FLAG. We also thank Dr. Xiaofei Li (Rhode Island Hospital, Cardiovascular Research Center) for help with confocal microscopy.

Author contributions—N. N. T., K. S. M., and K. R. conceptualization; N. N. T., K. S. M., K. R., B. C. B., A. Y. K., S. D., R. T., D. Y.-E. C., M. Z., A. X., and Y. L. data curation; N. N. T., K. R., B. C. B., A. Y. K., S. D., R. T., D. Y.-E. C., and A. X. formal analysis; N. N. T., K. S. M., A. K., and G. K. funding acquisition; N. N. T., K. R., R. T., M. Z., and Y. L. investigation; N. N. T., K. S. M., B. C. B., A. Y. K., S. D., D. Y.-E. C., M. Z., and A. X. methodology; N. N. T., K. S. M., K. R., and A. Y. K. writing–original draft; N. N. T., K. R., B. C. B., A. Y. K., S. D., R. T., M. Z., A. K., and G. K. supervision; A. K. and G. K. resources; G. K. project administration.

Funding and additional information—This work was supported by Grant 1059B191300954 from the Scientific and Technological Research Council of Turkey (to N. N. T.), National Institutes of Health Grants R01HL110791, R01HL134706, and R01HL139467 (to G. K.), and Rhode Island Foundation Grant 701-7131778 (to K. S. M.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: 3wRbCM, 3-week-old rabbit cardiomyocytes; APD, action potential duration; CMV, cytomegalovirus; CRU, Ca^{2+} release unit; HA, hemagglutinin; hrGFP, humanized R. reniformis GFP; LTCC, L-type calcium channel; MOI, multiplicity of infection; NRBcM, neonatal rabbit cardiomyocytes; PLA, proximity ligation assay; IP, immunoprecipitation; DAPI, 4′,6-diamino-2-phenylindole.

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