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Structural analyses of Ca^{2+} /CaM interaction with Na_v channel C-termini reveal mechanisms of calcium-dependent regulation

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Ca^{2+} regulates voltage-gated Na^+ (Na_v) channels, and perturbed Ca^{2+} regulation of Na_v function is associated with epilepsy syndromes, autism and cardiac arrhythmias. Understanding the disease mechanisms, however, has been hindered by a lack of structural information and competing models for how Ca^{2+} affects Na_v channel function. Here we report the crystal structures of two ternary complexes of a human Na_v cytosolic C-terminal domain (CTD), a fibroblast growth factor homologous factor and Ca^{2+} /calmodulin (Ca^{2+} /CaM). These structures rule out direct binding of Ca^{2+} to the Na_v CTD and uncover new contacts between CaM and the Na_v CTD. Probing these new contacts with biochemical and functional experiments allows us to propose a mechanism by which Ca^{2+} could regulate Na_v channels. Further, our model provides hints towards understanding the molecular basis of the neurologic disorders and cardiac arrhythmias caused by Na_v channel mutations.

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Voltage-gated Na⁺ (Na_V) channels underlie the rapid upstroke of action potentials. Mammalian Na_V channels are pseudotetramers with six-transmembrane segment repeats joined by intracellular linkers and flanked by intracellular N- and C-termini. The four repeats, each of which contains a voltage sensor, assemble to form a central pore. Although recent crystal structures of the tetrameric bacterial Na_V channel from *Arcobacter butzleri* (Na_VAb) provided detail about the pore and voltage sensors^{1,2}, Na_VAb tetramers lack the intracellular linkers and termini of mammalian Na_V channels. Those components are of particular interest because they confer isoform-specific regulatory effects, serve as sites of interaction for critical modulatory proteins and are loci for many disease-causing mutations.

The C-terminal domain (CTD) is of special interest because it exerts powerful effects upon channel inactivation³ and is the interaction site for several auxiliary proteins that modulate channel function, such as calmodulin (CaM) and fibroblast growth factor homologous factors (FHF), both of which regulate excitability through their CTD interactions^{4,5}. Moreover, many disease-causing mutations localize to Na_V CTDs or their associated proteins. Key examples include mutations in *SCN1A* and *SCN2A* (which encode the neuronal channels Na_V1.1 and Na_V1.2, respectively) that lead to various epilepsy syndromes, ataxia and autism^{6–8} or in the CTD of Na_V1.5, the cardiac Na_V channel encoded by *SCN5A*, which is a hotspot for mutations causing arrhythmias, cardiomyopathy and sudden infant death syndrome^{9–14}. Likewise, mutations in FHF or CaM have been associated with neurodegenerative disorders, cognitive deficits and arrhythmias^{14–16}. Structural information about Na_V CTDs,

however, has been limited. How the associated regulatory proteins influence channel function and how mutations in the CTDs or associated auxiliary proteins perturb channel function and at the molecular level are not well understood.

Among the proteins associated with Na_V CTDs, CaM is of particular interest because it acts as a sensor for Ca²⁺, which serves as a critical signal of electrical activity, providing powerful feedback regulation upon Na_V channel function¹⁷. Still, how Ca²⁺ and CaM affect Na_V channels have been controversial since sequence analysis of Na_V channels first revealed the presence of potential CaM-binding sites, including an ‘IQ’ motif⁵ and a potential Ca²⁺-binding site within the CTD¹⁸. Obtaining an understanding for CaM regulation of Na_V channels has been further complicated by apparent isoform-specific regulation. For example, CaM affects inactivation properties of the neuronal Na_V1.6 but not the skeletal muscle Na_V1.4 (ref. 19). For the cardiac Na_V1.5, CaM affects several different properties, including channel inactivation and persistent current^{20,21}. Nevertheless, the identification of disease-causing mutations within or near the Na_V IQ motifs of several Na_V isoforms^{6,20,22–24} highlight important roles for CaM. The potential significance of CaM-binding to Na_V channels has been further spotlighted by recent exome-sequencing studies in which searches for repeated rare variants or *de novo* mutations associated with autism identified *SCN1A* and *SCN2A* among the small list of loci^{25–27}; several of these catalogued Na_V mutations cluster in and around the IQ motif.

A major barrier to understand how Ca²⁺ and CaM act on Na_V channels has been that structural information is limited to Ca²⁺-free CaM (apoCaM) interacting with the CTD. While such

Figure 1 | Overall architecture of the ternary complexes containing a Na_V CTD, CaM and a FHF. (a) Sequence alignment of the Na_V1.2 and Na_V1.5 CTDs, with structural motifs and the IQ domain indicated. The interaction sites for the CaM N- and C-lobes and the CaM interlobular linker are also indicated. Human disease mutations in Na_V1.2 and Na_V1.5 clustering in the IQ domain are indicated in red; in blue are the homologous positions of disease mutations in Na_V1.1. (b) The Na_V1.2/Ca²⁺ structure containing the Na_V1.2 CTD (purple), FGF13 (silver) and CaM (blue). (c) The Na_V1.5/Ca²⁺ structure containing the Na_V1.5 CTD (orange), FGF12 (pink) and CaM (red). (d) Overlay of the Na_V1.2/Ca²⁺ and the Na_V1.5/Ca²⁺ structures aligned to the IQ motifs. (e) Overlay of the Na_V1.2/Ca²⁺ structure; the Na_V1.5/Ca²⁺ structure; and the Na_V1.5/-Ca²⁺ structure (Protein Data Bank accession code 4DCK), all aligned to the globular domain of the respective CTDs. For clarity, their respective CaM structures were omitted. This arrangement emphasizes the different angles between the globular domains and the IQ motifs among the three structures.

studies defined an interaction between the decalcified C-lobe of CaM and the IQ motif^{28–30}, those structures were unable to reveal how Ca^{2+} affects Na_V function and did not provide insight into mechanisms for IQ motif disease mutations, including a familial autism mutation in the neuronal $\text{Na}_V1.2$ channel⁷ and a cardiac arrhythmia mutation in the cardiac $\text{Na}_V1.5$ (ref. 20) that fall outside of the apoCaM contact sites.

Here we present crystal structures of $\text{Na}_V1.2$ and $\text{Na}_V1.5$ CTDs bound to Ca^{2+} /CaM. Comparison with our previous structure obtained with apoCaM reveals novel and unexpected Ca^{2+} /CaM contacts and stark differences in the overall conformation of the ternary complex, including a Ca^{2+} -dependent interaction between the CaM N-lobe and an extended helix that contains the IQ motif. Together, these findings provide a basis for understanding the effects of specific disease-causing mutations within Na_V CTD domains.

Results

Ternary complex structures of a Na_V CTD and FHF and Ca^{2+} /CaM. To define how Ca^{2+} regulates Na_V channels, we solved crystal structures of complexes containing a Na_V CTD, Ca^{2+} /CaM and a FHF. FHFs are constitutive Na_V subunits in the brain^{4,31} and heart³² and their inclusion allows us to compare Ca^{2+} /CaM structures with our previous complex containing apoCaM³⁰. We tested several different combinations of FHFs and Na_V CTDs with Ca^{2+} /CaM and eventually succeeded in crystallizing two ternary complexes: a 6xHis-tagged human $\text{Na}_V1.5$ CTD (amino acids 1,773–1,940), human FGF12B and CaM; and a 6xHis-tagged human $\text{Na}_V1.2$ CTD (amino acids 1,777–1,937), human FGF13U and CaM. The sequences of the Na_V CTDs are highly conserved among the subtypes (76% identities between $\text{Na}_V1.5$ and $\text{Na}_V1.2$, and 91% of the amino acids are conserved; Fig. 1a) and the solution structures of the proximal $\text{Na}_V1.2$ CTD and $\text{Na}_V1.5$ CTD are nearly identical^{33,34}. Likewise, FGF12B and FGF13U are highly conserved (69% identities), and their crystal structures in the absence of any binding partners are similar³⁵. Thus, we anticipated significant similarities between the $\text{Na}_V1.2$ - and $\text{Na}_V1.5$ -containing complexes.

Both complexes were expressed in *Escherichia coli* and purified in the presence of 2 mM Ca^{2+} by Co^{2+} affinity chromatography followed by size exclusion chromatography. The two ternary complexes (combined $M_w \sim 60$ kDa) were stable and each eluted in a single peak on a size exclusion column (Supplementary Fig. 1). Their individual profiles were highly similar to each other and to what we observed for the ternary complex containing the $\text{Na}_V1.5$ CTD, FGF13U and CaM purified in EGTA, for which we had demonstrated a stoichiometry of 1:1:1 (ref. 30).

The complex containing $\text{Na}_V1.2$ CTD, FGF13U and CaM (hereafter referred to as $\text{Na}_V1.2/\text{Ca}^{2+}$) was crystallized in the C2 space group with two copies of the ternary complex in each asymmetric unit. The crystals were grown in the presence of 2 mM Ca^{2+} and diffracted to 3.02 Å Bragg spacings. The experimental phases were derived by single anomalous dispersion from selenomethionine (SeMet)-substituted crystals and improved by twofold non-crystallographic averaging, which yielded a good-quality electron density map (Supplementary Fig. 2A). The final model contains the $\text{Na}_V1.2$ amino acids 1,788–1,929, FGF13U amino acids 11–158 and the CaM amino acids 7–149. The model was refined to $R_{\text{work}}/R_{\text{free}}$ of 21.5/24.6% (Table 1). The ternary complex containing $\text{Na}_V1.5$ CTD, FGF12B and CaM (hereafter referred to as $\text{Na}_V1.5/\text{Ca}^{2+}$) was crystallized in the P3₁21 space group with one copy of the ternary complex in each asymmetric unit. The crystals were grown in the presence of 2 mM Ca^{2+} and diffracted anisotropically to 3.8/5.4/6.0 Å Bragg spacings. Molecular replacement was performed to obtain the

phases (see Methods for the details). The final model contains the $\text{Na}_V1.5$ amino acids 1,786–1,927; FGF12B amino acids 12–152; and the CaM amino acids 7–148. Despite the resolution limit, the model refined to good statistics ($R_{\text{work}}/R_{\text{free}}$ of 26.2/31.8%) and good geometry (Table 1 and Methods for the refinement). 2Fo–Fc OMIT map shows a good-quality electron density, supporting the accuracy of the model given the resolution (Supplementary Fig. 2B). There is no significant difference in the refinement statistics when the data were truncated to 6.0 Å (Table 1).

Figure 1b,c shows the overall architecture of the $\text{Na}_V1.2/\text{Ca}^{2+}$ and $\text{Na}_V1.5/\text{Ca}^{2+}$ ternary complexes, respectively. In both complexes, the Na_V CTD comprises one globular domain that contains an EF-hand fold followed by an extended helix that contains the IQ motif. We refer to this helix as the IQ domain. The FHF binds to the CTD globular domain and CaM binds to the IQ domain. When the $\text{Na}_V1.5/\text{Ca}^{2+}$ and the $\text{Na}_V1.2/\text{Ca}^{2+}$ structures were superimposed with respect to their IQ domain, structural conservation was observed within the region containing the IQ domain and CaM (r.m.s.d. of 1.7 Å; Fig. 1d).

When the two structures (stripped of their respective CaM molecules) are superimposed relative to their respective CTD's globular domains together with FHFs, both Na_V CTD globular domains and FHFs are very similar to the r.m.s.d. of 0.89 Å, with their IQ domains out of register (Fig. 1e) because of different angles between their CTD's globular domain (along with FHFs) and their IQ domain. The different angle may reflect an isoform-specific structural difference or inherent flexibility between the two domains of Na_V CTD.

To focus on the Ca^{2+} -dependent conformational changes, we also superimposed our previously solved structure of a ternary complex of $\text{Na}_V1.5$ CTD, FGF13 and CaM crystallized in the absence of Ca^{2+} (ref. 30), hereafter referred to as $\text{Na}_V1.5/-\text{Ca}^{2+}$. With the CaM molecules stripped for clarity, Fig. 1e shows that the difference in angle between the respective Na_V CTD globular domains and the IQ domains is even more pronounced between $\text{Na}_V1.5/\text{Ca}^{2+}$ and $\text{Na}_V1.5/-\text{Ca}^{2+}$ than between $\text{Na}_V1.2/\text{Ca}^{2+}$ and $\text{Na}_V1.5/\text{Ca}^{2+}$ (Fig. 1e). Although it is possible that these rigid-body motions between the CTD globular domains and their respective IQ domains are associated with Ca^{2+} binding, these motions can also be due to inherent flexibility between these two domains. As the $\text{Na}_V1.2/\text{Ca}^{2+}$ structure provides higher resolution than the $\text{Na}_V1.5/\text{Ca}^{2+}$ structure and the differences are otherwise minimal, subsequent analyses focus only on the $\text{Na}_V1.2/\text{Ca}^{2+}$ structure.

A Ca^{2+} -dependent CaM N-lobe interaction with the IQ domain. The most significant effects of Ca^{2+} are the changes in the interactions between CaM and the respective IQ domains as shown in Fig. 2a,b, in which the $\text{Na}_V1.2/\text{Ca}^{2+}$ and $\text{Na}_V1.5/-\text{Ca}^{2+}$ structures are aligned by their IQ domains. The CaM in the $\text{Na}_V1.5/-\text{Ca}^{2+}$ structure adopts an extended conformation with the α -helical interlobular linker between the CaM/N- and C-lobes holding the N-lobe away from the C-lobe that binds to the proximal portion (IQ motif) of the IQ domain. NMR structures of Ca^{2+} -free CaM and an isolated IQ domain peptide from either $\text{Na}_V1.2$ or $\text{Na}_V1.5$ also showed an interaction between the CaM C-lobe and the IQ motif, but not the CaM N-lobe and the IQ domain^{28,29}. In our new $\text{Na}_V1.2/\text{Ca}^{2+}$ structure, in contrast, the α -helical interlobular linker is unwound, thereby allowing the Ca^{2+} -bound CaM N-lobe to envelope the distal portion of the IQ domain and provide additional contacts between the linker and the IQ domain and between the CaM N-lobe and the IQ domain that are not present in the absence of Ca^{2+} . These new contacts include residues mutated in channelopathies, as discussed below.

Superposition of $\text{Na}_V1.5/-\text{Ca}^{2+}$ and $\text{Na}_V1.2/\text{Ca}^{2+}$ structures shows that there are only minor conformational changes with

Table 1 | Data collection, phasing and refinement statistics.

	SeMet Na _V 1.2/CaM/FGF13U/Ca ²⁺	Na _V 1.5/CaM/FGF12B/Ca ²⁺	Na _V 1.5/CaM/FGF12B/Ca ²⁺ with 6 Å cutoff
Data collection			
Space group	C2	P3 ₁ 21	P3 ₁ 21
Wavelength (Å)	0.9792	1.0	1.0
Cell dimensions			
<i>a</i> , <i>b</i> , <i>c</i> (Å)	153.13, 86.11, 109.40	115.199, 115.199, 120.107	115.199, 115.199, 120.107
α , β , γ (°)	90.0, 100.9, 90.0	90.0, 90.0, 120.0	90.0, 90.0, 120.0
Resolution (Å)	50.00–3.02 (3.07–3.02)	50.00–3.80 (3.87–3.80)*	50.00–6.00 (6.10–6.00)
<i>R</i> _{sym} (%)	16.4 (48.3)	6.9 (39.2)	5.7 (24.1)
<i>I</i> / σ <i>I</i>	16.8 (1.5)	34.7 (1.8)	53.0 (7.6)
Completeness (%)	94.3 (66.6)	46.1 (13.2)	92.6 (61.5)
Redundancy	12.5 (5.6)	7.2 (2.2)	8.5 (6.5)
SAD phasing			
Figure of merit	0.30 (50.0–3.02 Å)		
Refinement			
Resolution (Å)	48.23–3.02 (3.09–3.02)	41.75–3.84 (5.00–3.54) [†]	41.75–6.00 (7.00–6.00)
Completeness (%)	91.8 (53.0)	40.53 (12.91)	92.89 (81.72)
No. of reflections	26,179	3,700	2,301
<i>R</i> _{work} /free	21.48/24.64	26.01/31.75	23.61/31.82
Ramachandran (%)			
Favoured	95.7	95.5	95.5
Outliers	0.4	0.5	0.5
R.m.s.d			
Bond lengths (Å)	0.011	0.011	0.012
Bond angles (°)	1.04	0.996	1.093

SAD, single anomalous dispersion; SeMet, selenomethionine.

 $R_{\text{sym}} = \sum |I| - \langle I \rangle / \sum I$, where $\langle I \rangle$ is the average intensity of symmetry-equivalent reflections. $R_{\text{work}} = \sum |F_o - F_c| / \sum |F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively. $R_{\text{free}} = R$ -factor calculated using a subset (5–8%) of reflection data chosen randomly and omitted throughout refinement. The figure of merit = $|F(\text{hkl})_{\text{best}}| / |F(\text{hkl})|$ and calculated using Phaser before density modification.

*The crystal diffracts anisotropically to 3.8/5.4/6.0 Å.

†The data treated with ellipsoidal truncation and anisotropic scaling were used for refinement (1).

respect to the CaM C-lobe. In both structures, the CaM C-lobe adopts the ‘semi-open’ conformation (Fig. 2c) that was first described for apoCaM bound to an IQ motif from an unconventional myosin³⁶. Significant conformational changes, however, occurred within the N-lobe of CaM, which assumes a ‘closed’ conformation in the Na_V1.2/Ca²⁺ structure (Fig. 2c,d), similar to the conformation observed for the N-lobe of Ca²⁺/CaM bound to its target peptide in CaMKII³⁷. For comparison, in the Na_V1.5/Ca²⁺ structure the unbound CaM N-lobe displays the ‘semi-open’ conformation³⁰. In addition, the arrangement of CaM lobes with respect to the IQ domain is novel to the best of our knowledge (see Discussion for further details).

These conformation differences of the individual CaM lobes in the Na_V1.2/Ca²⁺ structure suggested that the C-lobe was unlikely to be fully occupied, while the N-lobe is saturated with Ca²⁺. To test this hypothesis we collected data for the Na_V1.2/Ca²⁺ crystals at a long wavelength (1.55 Å), for which the anomalous scattering power of Ca²⁺ ($f' \sim 1.2$ e) is higher than that of sulphur (~ 0.6 e), while that of Mg²⁺ is nearly silent (~ 0.1 e). Although we observed strong anomalous difference Fourier peaks in the Ca²⁺-binding loops of the CaM N-lobes, only weak peaks were observed in the Ca²⁺-binding loops of the CaM C-lobe (Supplementary Fig. 3), suggesting that the affinity for Ca²⁺ in the CaM C-lobe of Na_V1.2/Ca²⁺ is low (compared with the CaM N-lobe) and that the CaM C lobe is only partially occupied with Ca²⁺, consistent with the semi-open conformation of the CaM C-lobe.

This newly discovered interaction between the CaM N-lobe and the distal C-terminal portion of the IQ domain is driven

mainly through van der Waals forces (Fig. 2e), burying Na_V1.2 hydrophobic side chains (Leu1,920, Leu1,921, Val1,925 and Val1,928) and has functional and disease-related implications. First, this additional interaction between the distal IQ domain and the CaM N-lobe offers an explanation for a previous report that an Ala1,924Thr mutation in the cardiac Na_V1.5 channel (equivalent to Val1928 in Na_V1.2) causes the life-threatening arrhythmia Brugada Syndrome and eliminates the Ca²⁺/CaM-dependent slow inactivation observed for the wild-type Na_V1.5 channel²⁰. We hypothesized that the Ala1924Thr mutation in the Na_V1.5 CTD affected the affinity for Ca²⁺/CaM and tested the hypothesis by isothermal calorimetry (ITC). Indeed, in the presence of saturating 5 mM Ca²⁺, the mutation reduced the affinity for Ca²⁺/CaM by approximately threefold compared with the wild-type Na_V1.5 CTD (Supplementary Fig. 4A and Table 2). In contrast, the affinity of the mutant Na_V1.5 CTD for CaM in the absence of Ca²⁺ was mildly increased (Table 2). Second, we found that the CaM N-lobe interaction with the distal IQ domain provides a significant boost to the affinity of Ca²⁺/CaM for the Na_V CTD. A previous report using ITC³⁸ had found the affinity of Ca²⁺/CaM for a Na_V1.5 peptide containing the IQ motif to be ~ 2.1 μM. Those thermodynamic parameters, however, were obtained in experiments employing an IQ domain in which the newly discovered CaM N-lobe contact site is truncated. We therefore performed ITC with a longer Na_V1.5 CTD (through amino acid 1,940) and observed a significantly higher affinity for both Ca²⁺/CaM and apoCaM (~ 100 nM, as shown in Supplementary Fig. 4A,B and Table 2). To assure that the lower K_d values we obtained were indeed

Figure 2 | Ca^{2+} -dependent calmodulin interactions. (a) Overlay of the $\text{Nav1.2}/\text{Ca}^{2+}$ and the $\text{Nav1.5}/\text{-Ca}^{2+}$ structures, both aligned to their IQ domains. Colours for Nav1.2 : Nav1.2 CTD (purple), FGF13 (silver) and CaM (blue). Colours for Nav1.5 : Nav1.5 CTD (pale green), CaM (dark green), FGF12 (lime). (b) Zoomed-in view of the IQ domains and CaM with the same orientation as a. Ca^{2+} ions are shown as yellow balls. Ca^{2+} -Free-CaM from the $\text{Nav1.5}/\text{-Ca}^{2+}$ structure is coloured green and Ca^{2+} -CaM from the $\text{Nav1.2}/\text{Ca}^{2+}$ structure is coloured blue. (c) $\text{C}\alpha$ trace overlay of the Ca^{2+} -loaded CaM N-lobes (left) from the $\text{Nav1.2}/\text{Ca}^{2+}$ structure (blue); and Ca^{2+} -CaM bound to the CaM-binding peptide in CaMKII (PDB ID 3GP2, red); and the Ca^{2+} -free CaM C-lobes (right) from the $\text{Nav1.2}/\text{Ca}^{2+}$ structure (blue); and CaM bound to the IQ motif in scallop myosin (PDB ID 2IX7, red). (d) Stereo view of the coordination geometry of the two Ca^{2+} -binding sites in the CaM N-lobe within the $\text{Nav1.2}/\text{Ca}^{2+}$ structure (top) with the Fo-Fc omit map for Ca^{2+} ions (magenta, 6σ) and 2Fo-Fc omit map (bottom, salmon, 1σ). W stands for the water. (e) Zoomed-in view of the CaM/N-lobe (blue) interactions with $\text{Nav1.2}/\text{Ca}^{2+}$ (purple). Arrow points to Val1928, equivalent to Ala1924 in Nav1.5 .

because of the longer CTD and not because of technical differences, we measured the affinity of CaM for the Nav1.5 CTD truncated at amino acid 1,924, for which we obtained a value of $2.0 \pm 0.4 \mu\text{M}$ in 5 mM Ca^{2+} (Table 2 and Supplementary Fig. 4B). This result is in excellent agreement with the value previously obtained³⁸, thereby allowing us to benchmark our

thermodynamic data against that report. Thus, the ~ 20 -fold higher affinity for CaM obtained with the longer CTD highlights critical contributions of the more distal IQ domain residues. Comparison of the N values of interactions for $\text{Ca}^{2+}/\text{CaM}$ with the two CTDs by ITC (Table 2) further demonstrates the importance of the more distal residues in the IQ domain. For the

Table 2 | Thermodynamic parameters for CaM interaction with the Nav1.5 CTD.

Titrant	N	Cell	K_d (nM)	ΔH (kcal mol ⁻¹)	ΔS (cal mol ⁻¹ deg ⁻¹)	N-value
Ca ²⁺ /CaM	3	1,773-1,924	1,990 ± 358*	-3.77 ± 0.27	12.4 ± 0.5	0.38 ± 0.02
apoCaM	4	1,773-1,940	88 ± 6*	-10.69 ± 0.06	-4.2 ± 0.3	0.94 ± 0.03
Ca ²⁺ /CaM	3	1,773-1,940	132 ± 9	-9.94 ± 0.33	-2.4 ± 0.1	0.83 ± 0.05
Ca ²⁺ /CaM	3	1,773-1,940 (A1924T)	497 ± 32*	-7.81 ± 0.09	2.1 ± 0.2	0.42 ± 0.02
apoCaM	3	1,773-1,940 (A1924T)	61 ± 4*	-11.16 ± 0.14	-5.1 ± 0.3	0.70 ± 0.01

CaM, calmodulin; CTD, C-terminal domain.

*P < 0.05 compared with Nav1.5 CTD (amino acids 1,773-1,940) plus Ca²⁺/CaM.

shorter CTD, the *N* value of the Ca²⁺/CaM-CTD interaction is close to ~0.5 (0.38 in our measurement, and 0.56 previously reported³⁸), suggesting that one Ca²⁺/CaM can bind two CTDs. The *N* value we obtained for the longer CTD was doubled (0.84), suggesting that one Ca²⁺/CaM binds one CTD, and fits well with our crystallographic observation that both N- and C-lobes simultaneously bind to different sites within the IQ domain of Nav CTD. Consistent with our observation, *N* value of the interaction between Ca²⁺/CaM and Ala1924Thr of the longer CTD is reduced to half of the wild type (0.42; Table 2). Thus, these data underlined the importance of the interaction between CaM and the more distal region of the IQ domain and supported a model in which there are simultaneous interactions of the two CaM lobes (at different sites on the IQ domain).

Ca²⁺-dependent changes and disease mechanisms. While the new CaM N-lobe contact with the Nav1.2 CTD is the most obvious Ca²⁺-dependent structural change, we also identified additional new Ca²⁺/CaM contact sites within the Nav1.2 IQ domain, which may provide insight for several other Nav channelopathies. For example, Arg1918 (Fig. 3a,b), which contacts Asp79 and Ser82 in the CaM interlobular linker in the Ca²⁺-loaded complex (but not in the apoCaM structure), was reported in a patient with febrile seizures and childhood absence epilepsy²³, and mutation of the equivalent Arg1928 in the homologous Nav1.1 was found in a patient with severe myoclonic epilepsy of infancy²⁴.

In addition, of particular interest was a familial autism mutation Arg1902Cys in Nav1.2 (ref. 7) especially since recent analyses of rare *de novo* mutations in subjects with autism have identified SCN2A as one of a handful of high-confidence autism spectrum disorder genes^{25,39}. Arg1902 sits at the hinge between the CTD globular domain and the IQ domain helix (Fig. 3c). We had previously found that the Arg1902Cys mutation conferred a Ca²⁺/CaM-dependent conformational change indicated by a significant Ca²⁺-dependent shift in migration on a size exclusion column of a Nav1.2 CTD/CaM binary complex that was not observed with the wild-type complex²¹. Although Arg1902 (or the Arg1898 equivalent in Nav1.5) does not make any direct contacts with CaM either in the presence or in the absence of Ca²⁺, it interacts with the side chain of Glu1905 (Glu1901 in Nav1.5) one turn below along the IQ domain helix and also forms cation- π interaction with the side chain of Tyr98 in FGF13U in the Nav1.5/-Ca²⁺ structure. Tyr98 in FGF13 and Glu1905 in Nav1.2 (Glu1901 in Nav1.5) also interact with Lys95 within the third CaM EF-hand in the CaM C-lobe. Interestingly, Glu1905 in Nav1.2, Tyr98 in FGF13U and Lys95 in CaM are the only residues within the ternary complex that make interactions with each of the other partners. Thus, we suspected that the relayed interactions from Lys95 (CaM) through Glu1905 (Nav IQ domain) to Arg1902 (Nav globular domain), stabilized by the cation- π interaction with Tyr98 in FGF13, suppress the Ca²⁺-dependence of Nav1.2 channels and that disruption of these interactions would affect Ca²⁺-dependent

regulation of Nav1.2 channels (Fig. 3c). We tested this hypothesis in two ways.

First, we expressed either wild-type or Arg1902Cys-mutant Nav1.2 along with FGF14, the best characterized neuronal FHF^{4,31,40} in HEK293 cells, in which endogenous CaM is abundant, and recorded Na⁺ currents in the presence of saturating internal Ca²⁺ or nominally zero internal Ca²⁺ (Supplementary Fig. 5). For wild-type Nav1.2, inclusion of 10 μ M Ca²⁺ in the patch pipette did not affect peak current density, the $V_{1/2}$ of activation or the $V_{1/2}$ of steady-state inactivation. In contrast, for the Arg1902Cys mutant the addition of Ca²⁺ induced a large approximately -10 mV shift in the $V_{1/2}$ of steady-state activation and inactivation (Fig. 3d, Supplementary Fig. 6A and Supplementary Table 1). To test further our hypothesis that disruption of the Nav CTD to CaM relay affected Ca²⁺-dependent regulation of Nav1.2, we ablated the key intermediary by mutating Nav1.2 Glu1905 to Gln. Recordings from the Glu1905Gln-mutant channels phenocopied those from the Arg1902Cys-mutant channels (Supplementary Fig. 6A and Supplementary Table 1). Second, to test the proposed role of Arg1902 in the relayed interactions in Nav1.2 CTD/apoCaM, we measured the affinity of apoCaM for the wild-type and Arg1902Cys-mutant Nav1.2 CTDs by ITC. Consistent with our hypothesis, the Arg1902Cys mutation reduced affinity of apoCaM for the Nav1.2 CTD significantly (Table 3 and Supplementary Fig. 4C). We did not observe a difference in affinity for Ca²⁺/CaM between the wild-type and the Arg1902Cys-mutant CTD (Table 3 and Supplementary Fig. 4D). As our ITC measurement in the presence of Ca²⁺ could be complicated by an additional binding process because of Ca²⁺-loading of the CaM C-lobe, which is essentially unoccupied in the Nav1.2/+Ca²⁺ structure (Supplementary Fig. 3), we therefore prepared CaM in which the third and fourth EF hands were mutated to ablate Ca²⁺ binding to the CaM C-lobe⁴¹ and repeated the ITC measurements with this crippled CaM₃₄ mutant. The ITC experiment with the CaM₃₄ mutant showed a reduced affinity for the Arg1902Cys mutant compared with the wild CTD in the presence of Ca²⁺ (Table 3 and Supplementary Fig. 4E). Thus, these data are consistent with the previously observed shift in mobility on gel filtration of the Arg1902Cys/CaM binary complex²¹ and suggest that by disrupting the relayed interactions to CaM the familial autism Nav1.2 Arg1902Cys mutation revealed Ca²⁺-dependent effects upon channel function that were suppressed in the wild-type channel.

We next investigated whether the Ca²⁺-dependent interaction of the CaM N-lobe was required for the Ca²⁺-dependent regulation of Nav1.2 exposed by the Arg1902Cys mutation. We mutated Val1925, one of the Nav1.2 hydrophobic side chains buried by the calcified CaM N-lobe (see Fig. 2e), to Lys and examined how this mutant affected the Ca²⁺-dependent shift in activation and steady-state inactivation in the context of the Arg1902Cys mutant. With this additional Val1925Lys mutation, the Arg1902Cys mutant no longer displayed any Ca²⁺-dependent effects on either activation or steady-state inactivation

Figure 3 | Ca²⁺-dependent structural changes highlight mechanisms for Nav channel dysfunction with disease mutations. (a) Overall structure of the Nav_v1.2/Ca²⁺ and Nav_v1.5/-Ca²⁺ complexes for orientation, as in Fig. 2a. Boxed area shows the relative region for focus in b,c. (b) Zoomed view of the Nav_v1.5/-Ca²⁺ and Nav_v1.2/Ca²⁺ structures demonstrating the Ca²⁺-dependent changes in interaction between the Nav CTD and the CaM C-lobe and CaM interlobular linker. For comparison, the labelling for the Arg residues within the Nav_v1.5 CTD employs the corresponding numbers for Nav_v1.2; and the Nav_v1.5 numbers are shown in parentheses. (c) Zoomed view of the Nav_v1.5/-Ca²⁺ structure focusing on the interaction between Arg 1898 (Arg 1902 in Nav_v1.2) in the Nav_v1.5 CTD and Lys 95 in CaM via Glu1901 (Glu1905 in Nav_v1.2) and Tyr98 in FGF13. As in b, the numbering within the CTD corresponds to the Nav_v1.2 sequence and the Nav_v1.5 equivalents are in parentheses. (d) Activation and steady-state inactivation relationships in 0 mM Ca²⁺ or 10 μM-free Ca²⁺ in the recording pipette for wild-type Nav_v1.2 or the Arg1902Cys CaM N-lobe interaction mutant. Summary data and statistics are provided in Table 1.

(Supplementary Fig. 6B and Supplementary Table 1), thereby implicating a requirement for the interaction between the calcified CaM N-lobe and Nav_v1.2. When analysed independently, Val1925Lys did not expose any Ca²⁺-dependent regulation (Supplementary Fig. 6B and Supplementary Table 1).

Ca²⁺ binding is restricted to CaM. Our analyses suggested that Ca²⁺-dependent regulation of Nav channel function derives from Ca²⁺-dependent changes in the interaction between CaM and the Nav CTD, yet it has previously been suggested that Ca²⁺ also affects Nav channels by binding directly to an EF-hand motif within the Nav CTD influences Nav channel function^{18,34,42}.

To query whether Ca²⁺ can bind directly to the Nav CTD EF-hand motif, we used our anomalous scattering studies. Even though we detected anomalous difference signals from many sulphur atoms (in methionines) whose signal is approximately twofold weaker than that for Ca²⁺, we did not detect anomalous difference signal for Ca²⁺ within the proposed Ca²⁺-binding loops (Fig. 4a) and we observed a strong signal for Ca²⁺ bound to the CaM N-lobe (Supplementary Fig. 3). Comparing the acidic and polar residues proposed to coordinate Ca²⁺ in the putative Nav CTD EF-hand¹⁸ with those in the Ca²⁺-binding EF hands in the CaM N-lobe of the associated CaM provides an explanation. While both EF hands of the Ca²⁺-loaded CaM N-lobe contain a sufficient number of acidic and polar residues

Table 3 Thermodynamic parameters for CaM interaction with the Na _v 1.2 CTD.						
Titrant	N	Cell	K _d (nM)	ΔH (kcal mol ⁻¹)	ΔS (cal mol ⁻¹ deg ⁻¹)	N-value
apoCaM	3	WT	36 ± 6	− 4.54 ± 0.1	18.6 ± 0.2	1.02 ± 0.02
Ca ²⁺ /CaM	3	WT	1,713 ± 333	6.01 ± 0.5	47.0 ± 1.3	0.76 ± 0.02
Ca ²⁺ /CaM ₃₄	3	WT	388 ± 22	− 13.85 ± 0.4	− 17.9 ± 1.4	0.86 ± 0.05
apoCaM	3	R1902C	958 ± 80*	− 3.28 ± 0.3	− 2.1 ± 0.2	0.58 ± 0.13
Ca ²⁺ /CaM	3	R1902C	1,510 ± 187	6.19 ± 0.9	48.0 ± 2.8	0.74 ± 0.03
Ca ²⁺ /CaM ₃₄	3	R1902C	2,931 ± 123*	− 12.53 ± 0.9	17.4 ± 2.8	0.49 ± 0.02

CaM, calmodulin; CTD, C-terminal domain; WT, wild type.
*P<0.05 compared with WT.

Figure 4 | The Na_v CTD EF hand does not bind Ca²⁺. (a) Anomalous difference Fourier map for two CaMs in the asymmetric unit from the Na_v1.2 CTD crystal. The map was calculated using data from 25.0 to 5.5 Å of the native crystal using the final model phases. The anomalous difference peaks, coloured in green mesh, are contoured at 2.8σ. The arrows indicate the expected positions of Ca²⁺ in CaM. Side chains of methionines are shown. (b) Comparison of the first and second Ca²⁺-binding loop in the CaM/N-lobe with the EF hand motif from the Na_v1.2 CTD. Side chains involved in Ca²⁺ coordination in the Ca²⁺-binding loops of EF hands are shown. Ca²⁺ is shown as a green ball.

(a total of five) positioned to coordinate Ca²⁺ in an optimal geometry (Fig. 4b and see Fig. 2d), the Na_v CTD EF hand does not (Fig. 4b). Thus, we conclude that the Na_v CTD EF hand does

not likely bind Ca²⁺; Ca²⁺-dependent effects on Na_v channel function are more likely mediated via CaM.

Discussion

Whether and how Ca²⁺ contributes to the regulation of voltage-gated Na_v currents has been a focus of significant controversy since potential CaM-binding sites were first identified within Na_v CTDs⁵. Our new structural model with Ca²⁺/CaM bound to the Na_v1.2 CTD, in context with previous structures demonstrating the interaction of apoCaM with various Na_v CTDs^{28–30}, reveals novel and unexpected interactions between the Ca²⁺-loaded CaM and the Na_v CTD. The apoCaM structures showed that the CaM C-lobe is anchored to the signature IQ motif within the extended IQ domain. On the basis of our new structural, biochemical and functional data, we propose that a major action of Ca²⁺ is to induce a conformational switch in the anchored CaM so that the CaM N-lobe swings into contact with the distal IQ domain, while the Ca²⁺-free CaM C-lobe remains anchored to the IQ motif (Fig. 2b). Interestingly, the new contact site for the calcified CaM N-lobe sits within a previously identified peptide that, when isolated from the adjacent IQ motif peptide, could only bind Ca²⁺-loaded CaM, in contrast to the IQ motif that supported apoCaM binding over Ca²⁺/CaM binding⁵. In addition, we observed that Ca²⁺ induces rearrangements between the Na_v CTD and the CaM intralobular linker, and between the Na_v CTD and the CaM C-lobe (Fig. 3b). We hypothesize that, together, these conformational changes may be propagated to the adjacent domain IV (DIV) transmembrane region of the channel to thereby affect Na_v function in an isoform-specific manner. Since our structures do not contain the transmembrane region of the channel, our model cannot explain how the conformational changes propagate to DIV. However, it is known that the conformational change of DIV voltage sensor (S4) is the rate-limiting step for channel inactivation^{43,44}. Thus, the Na_v CTD is in an advantageous position to affect channel gating.

Interestingly, the specific Ca²⁺/CaM-dependent effects appear to vary among different Na_v channels¹⁹. Our data add to that concept in which we found that the wild-type Na_v1.2 channel, not previously studied, was insensitive to Ca²⁺/CaM for the parameters we studied at either nominally zero or saturating (~10 μM) intracellular Ca²⁺. While the specific concentrations of internal Ca²⁺ studied here are outside the range of physiologic Ca²⁺ in neurons, these two levels allowed us to explore the bounds of Ca²⁺, and correlate to our structures, obtained in the absence or presence of Ca²⁺. These functional studies were also performed in the presence of a FHF, which was a component of the crystallized ternary complexes. Whether FHFs influence the Ca²⁺ dependence of Na_v currents has not yet been analysed. However, their inclusion in the functional studies is appropriate not only because of their presence in the crystal structures but also because of growing evidence that FHFs are important regulators of Na_v currents in the neurons and

cardiomyocytes^{4,16,32,45} in which Na_V1.2 and Na_V1.5 are expressed.

Nevertheless, the familial autism mutation Arg1902Cys introduced a large Ca²⁺-dependent shift in both channel activation and steady-state inactivation (Fig. 3 and discussed below). A gain-of-function effect of a channelopathic mutation is reminiscent to the mechanism by which mutations in Na_V1.5 lead to Long QT Syndrome^{9,10} and in some Na_V1.2 mutations associated with epilepsy⁴⁶. Combined with analysis of the Na_V1.2/Ca²⁺ structure, our functional data suggest that the relayed interactions from Lys95 (CaM) through Glu1905 (Nav IQ domain) to Arg1902 (Nav globular domain) mask a Ca²⁺-dependent shift in wild-type Na_V1.2 that is revealed by the Arg1902Cys familial autism mutant when the relay is disrupted. It is noteworthy that an Asp96Val mutation adjacent to Lys95 in CaM, recently reported in a patient with an arrhythmia syndrome, was also associated with moderate cognitive impairment¹⁴.

Taken together, we suggest that similar Ca²⁺-induced conformational changes of CaM in both Na_V1.2 and Na_V1.5 (interactions of the CaM N-lobe to the distal IQ domain of Na_V CTD) might be responsible for Ca²⁺-dependent regulation, and that their functional effects are isoform-specific. The concept that Ca²⁺-dependent regulation may be isoform-specific is consistent with a recent report showing that a rapid increase in intracellular Ca²⁺ diminished transient Na_V currents through Na_V1.4, but not through the Na_V1.5 isoform⁴⁷. With regard to Na_V1.2, only Arg1902Cys or Glu1905Gln unveiled a Ca²⁺-dependent functional effect. We reasoned that there are possible reasons for these isoform-specific differences of Ca²⁺ dependence. First, in the context of the full-length channels, it is possible that the conformational changes at the CTD could propagate to the DIV transmembrane region of the channel differently depending on the isoforms. The apparent isoform-specific difference in the angle between the Na_V globular domain and the extended IQ domain reported herein (Fig. 1e) provide one possibility. For example, perhaps the difference in the angle within Na_V1.2 masks the Ca²⁺-dependent changes in Na_V1.2 wild-type channel function initiated by the Ca²⁺-dependent CaM N-lobe interaction. Second, it is possible that there are unexamined functional parameters that are more relevant to Ca²⁺-dependent regulations that are less-isoform-specific. Third, it is possible that Ca²⁺/CaM does not mediate the observed Ca²⁺-dependent changes in functions of Na_V1.2 Arg1902Cys or Glu1905Gln. However, the fact that Val1925Lys mutation (in the background of Arg1902Cys) abolishes the Ca²⁺-dependent functional effect eliminates this possibility, as Val1925Lys would disrupt the binding of the CaM N-lobe to the distal IQ motif of Na_V1.2.

While mutagenesis studies have yielded suggestions about how Ca²⁺/CaM regulates Na_V channel function^{18,19,21,38,42,48,49}, human disease mutations can be particularly revealing. By identifying new Ca²⁺/CaM-dependent contacts with the Na_V CTD and demonstrating Ca²⁺-dependent conformation changes within the complex, our data provide a context in which to consider the effects of disease mutations that affect Na_V CTD–CaM interaction. Several epilepsy mutations in Na_V1.1 or Na_V1.2 are in residues that make different contacts with Ca²⁺/CaM compared with apoCaM (Fig. 3) as are additional Na_V1.1 and Na_V1.2 mutations associated with sporadic and familial cases of autism^{7,25,26}. Further, our analysis of the effects of the Na_V1.2 Arg1902Cys familial autism mutation demonstrates that disruption of the wild-type interaction between the CaM C-lobe and the CTD induces a Ca²⁺-dependent change for the mutant Na_V1.2 channel function. The marked hyperpolarizing shift in both Na_V1.2 activation and inactivation induced by the

Arg1902Cys autism mutation would affect neuronal excitability in Na_V1.2-expressing neurons, thus leading to an imbalance between excitation and inhibition known to drive neuropsychiatric phenotypes⁵⁰. In addition, our identification of the Ca²⁺-dependent CaM N-lobe interaction with the distal IQ domain, not predicted by previous structural studies, provides a molecular mechanism for the Ca²⁺-dependent dysfunction of the Na_V1.5 Ala1924Thr Brugada Syndrome mutation²⁰. Finally, our data provide a potential mechanism for the recently described mutations in CaM associated with arrhythmias and cognitive deficits¹⁴.

The Ca²⁺-loaded ternary complexes present several unusual and novel features for a CaM-containing complex. Among these are the dissimilar conformations of the CaM N-lobe and CaM C-lobe when calcified CaM is bound to the IQ domain (Fig. 2c). While the different conformations of the individual CaM lobes in our structures mirror the CaM lobe conformations seen in the SK K⁺ channel structure, the interactions between CaM and its target peptide(s) are markedly different. Within the SK K⁺ channel homotetramer, the calcified CaM N-lobe wraps around one helix from the C terminus of a protomer but the apoCaM C-lobe interacts with two helices from a different protomer within the tetramer⁵¹. Split roles for CaM lobes have also been suggested for Ca²⁺-dependent regulation of Ca_v channels^{52–54}, with one lobe responsible for mediating changes to global Ca²⁺, while the other responds to changes in local Ca²⁺. Comparison of our structures with Ca_v Ca²⁺ channel-derived structure provides an interesting contrast in structure and mechanisms by which Ca²⁺ regulates ion channel function, particularly since voltage-gated Na⁺ channels and Ca²⁺ channels are similar in sequence within their proximal CTDs. The similarities include not only the IQ domain to which CaM binds but also an EF hand motif in the Ca_v1.2 L-type Ca²⁺ channel that was hypothesized to serve as a site for Ca²⁺-dependent regulation⁵⁵ but (similar to the EF hand in the Na_V CTD) has also never been shown to bind Ca²⁺ with a physiologically meaningful affinity. In spite of these similarities in sequence, structures of the Ca_v CTDs bound to CaM are surprisingly different from what we observe for the Na_V CTDs. Foremost among these differences is that the Ca_v CTD/CaM complexes crystallized as a dimer of CTDs, and each CTD interacted with two CaM molecules for an overall stoichiometry of four CaM and two CTDs^{56,57}. Whether the dimerization of channels observed in the structure is functionally relevant has been debated. Nevertheless, the 2:1 stoichiometry between CaM and the Ca_v1.2 CTD contrasts markedly with the 1:1 stoichiometry between CaM and a Na_V CTD. With these differences, it is not surprising that the overall fold of the Ca_v1.2 CTD does not resemble the Na_V CTDs.

Even focusing specifically on the interactions between Ca²⁺/CaM and the respective IQ domains, the structures reveal stark differences (Fig. 5a–c). First, the arrangement of CaM with respect to the Na_V IQ domain is very different than Ca_v IQ domains. In both Ca_v1.2 and Ca_v2.1 IQ domains, CaM wraps around the IQ motif in a right-handed helical manner, regardless of the relative orientation of the IQ motifs (parallel or antiparallel to CaM lobes). On the contrary, CaM wraps around the Na_V IQ domain in a left-handed helical manner. Second, the overall Ca²⁺/CaM footprint on the Na_V IQ domain is longer. The signature IQ motif in Ca_v channels forms a central anchor for Ca²⁺/CaM interactions with both CaM lobes, while the IQ motif residues in the Na_V structures form contacts mostly with the CaM's Ca²⁺-free C-lobe. Thus, the Ca²⁺/CaM N-lobe is located further towards the C terminus on the Na_V IQ domains compared with the Ca_v IQ domains. A search of the Dali database⁵⁸ suggesting that the CaM conformation in the Na_V1.2/Ca²⁺ and Na_V1.5/Ca²⁺ structures is novel.

Figure 5 | CaM conformations when bound to Nav channels compared with Cav channels and CaMKII. (a–e) Unique arrangement of CaM relative to the IQ domains in the Nav1.5/ Ca^{2+} structure (a), the Nav1.2/ Ca^{2+} structure (b); a Cav1.2 structure (c, PDB ID: 2BE6); a Cav2.1 structure (d, PDB ID: 3DVM) and autoinhibitory (CaM binding) peptide in CaMKII (e, PDB ID: 1CDM). CaM N-lobe and C-lobe are coloured yellow and green, respectively, and the orientation of IQ motifs or autoinhibitory peptide is indicated with a colour gradient (N terminus blue and C terminus red). The positions of IQ(M) amino-acid residues on the IQ domains or the $^{302}\text{Al}^{303}$ amino-acid residues in the CaMKII autoinhibitory peptide are demarcated with two black lines. The cartoon figures are shown below each structure to illustrate the difference of the CaM–Nav1.2 interactions. CaM wraps around the Nav IQ domains in the left-handed manner in contrast to the right-handed wrapping seen in Cav structures and in CaMKII.

Together with this novel interaction mode between CaM and the Nav1.2 CTDs, our structural, biochemical and functional data provide a new framework for understanding how CaM affects Nav channels in physiology and disease.

Methods

Molecular biology. The following plasmids, for protein expression and purification, have been previously described: for crystallization the human Nav1.5 CTD (amino acids 1,773–1,940) and Nav1.2 (amino acids 1,777–1,937) were cloned into pET28 (Novagen)²¹; the human FGF13U (accession no. NM_033642) and FGF12B were cloned into the second multiple cloning site of pETDuet-1 (Novagen)⁵⁹; and CaM was cloned into pSGC02 (ref. 21). For isothermal titration calorimetry, human Nav1.5 CTD amino acids 1,773–1,924, Nav1.5 CTD amino acids 1,773–1,940 (and the Ala1924Thr mutant) were cloned into pET28. For electrophysiology, Nav1.2 was in pcDNA3.1 and FGF14b has previously been described⁶⁰. Site-directed mutagenesis was performed with QuikChange (Stratagene).

Recombinant protein expression and co-purification. The three plasmids for His6-Nav1.5 CTD, FGF12B and CaM or His6-Nav1.2 CTD, FGF13U and CaM were co-electroporated into BL-21 (DE3) cells. Proteins were grown in LB medium or M9 medium as described³⁰. Cells were harvested and resuspended in 300 mM NaCl, 20 mM Tris-HCl, 5 mM imidazole, 2 mM CaCl_2 , pH 7.5, supplemented with EDTA-free protease inhibitor mixture (Roche). The initial purification protocol has been previously described³⁰. Additional purification was performed using gel filtration on a Superdex 200 10/300I column on an AKTA FPLC (GE Healthcare) in 300 mM NaCl, 20 mM Tris-HCl, 5 mM imidazole, with 2 mM CaCl_2 , pH 7.5. Protein concentrations were determined using UV absorbance with Thermo NANODROP and were concentrated to $A_{280} = 12$ in above buffer for crystallization. For ITC experiments, the single plasmid was electroporated into BL-21 (DE3) cells and the proteins were expressed after induction with 1 mM isopropyl-1-thio- β -D-galactopyranoside for 16 h at 20 °C. CaM protein was purified as previously described⁴¹.

Crystallization and structure determination. Crystals were grown by vapour diffusion with the sitting-drop method. His6-Nav1.5 CTD, FGF12B and Ca^{2+} /CaM crystals were obtained with 20% PEG3350, 0.18 M MgSO_4 , 0.1 M sodium iodide and 2 mM CaCl_2 . His6-Nav1.2 CTD, FGF13U and CaM SeMet incorporated protein crystals were obtained with 14% pEG3350, 300 mM sodium acetate, 50 mM Tris pH 7.5 and 2 mM CaCl_2 . Before flash-freezing in liquid nitrogen, the crystals were cryoprotected by gradually increasing the concentration of glycerol in the well solution to 20%.

Crystals of SeMet-substituted Nav1.2 in complex with Ca^{2+} /CaM and FGF13 diffract to 3.02 Å Bragg spacings with the space group C2 and crystals of human Nav1.5 CTD in complex with Ca^{2+} /CaM and FGF12B diffract to 3.8 Å/5.4 Å/6 Å with the space group P3₁21 (Table 1). As for the structure determination of the complex of Nav1.2 CTD, Ca^{2+} -CaM and FGF13U, experimental phases were obtained from the SeMet-substituted complex using single anomalous dispersion. After initial automatic model building, model building was completed manually. The final model contains two complexes in the asymmetric unit and is refined to R/R_{free} of 21.5/24.6% with good geometry (Table 1). The model contains residues

1,788–1,929 of Nav1.2. As for the structure determination of the complex of Nav1.5, Ca^{2+} -CaM, and FGF12B, molecular replacement was performed to obtain the phases. In brief, the EF hand domain (residue 1,786–1,896) together with FGF13 from the previous Nav1.5 CTD structure in complex with FGF13U structure and Mg^{2+} -CaM (PDB ID:4DCK), IQ domain (residue 1,899–1,919) from the same complex structure, and the C-lobe of Mg^{2+} -CaM (residue 85–145) were used as independent search models and used to find the solutions using Phaser⁶¹. After the solution was found, the N-lobe of CaM was manually placed using $F_o - F_c$ map. Low-resolution structure refinement was performed using the reference model as the complex of Nav1.2, Ca^{2+} /CaM and FGF13. The serious anisotropy of amplitudes were corrected using the UCLA Diffraction Anisotropy Server (<http://services.mbi.ucla.edu/anisotropy>)⁶² and was used for the refinement. The final model contains one complex in the asymmetric unit and is of good quality with R/R_{free} of 26.0/31.8% (Table 1).

Isothermal titration calorimetry. Experiments were performed with an ITC-200 (MicroCal) at 20 °C. The solutions containing the wild-type Nav1.5 CTD, Nav1.2 CTD, Nav1.2 CTD R1902C mutation, Nav1.5 CTD amino acids 1,773–1,924, truncation mutant or A1924T mutant (25–35 μM) were titrated with one injection of 5 μl and 27 injections of 10 μl of solutions containing CaM or CaM₃₄ (240–310 μM). ITC experiments were repeated with different preparations and different concentrations at least three times to confirm thermodynamic parameters and stoichiometry values. The binding isotherms were analysed with a single site-binding model using the Microcal Origin version 7.0 software package (Originlab Corporation), yielding binding enthalpy (ΔH), stoichiometry (n), entropy (ΔS) and association constant (K_a). Results are presented as mean $\pm$ s.e.; statistical significance was assessed using a two-tailed Student's t -test and was set at $P < 0.05$.

Electrophysiology. Human embryonic kidney (HEK) 293 T cells (ATCC) were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum. The cells were plated on 60-mm tissue culture dishes and grown to 65–75% confluence, and then transfected using Lipofectamine 2000 (Invitrogen) with a total 6 μg of cDNAs encoding Nav1.2, FGF14b, $\beta 1$ and $\beta 2$ at a ratio of 2:1:1. One day after transfection, the cells were re-plated on coverslips coated with 50 $\mu\text{g ml}^{-1}$ poly-D-lysine (Sigma) for electrophysiological recordings. Transfected cells were identified by green fluorescent protein fluorescence. Na^+ currents were recorded using the whole-cell patch-clamp technique at room temperature (20–22 °C) 48–72 h after transfection. Electrode resistance ranged from 2.5 to 2.5 M Ω . Currents were filtered at 2.9 kHz and digitized at 20 Hz using an EPC 10 USB patch amplifier (HEKA Elektronik). Cells were allowed to stabilize for 7–10 min after the whole-cell configuration was established. Cells expressing peak current amplitude $> 6,000$ pA were excluded from kinetic analyses because of suboptimal voltage control, as were cells exhibiting peak current amplitudes < 600 pA to avoid contamination by endogenous currents. All cells were included in analyses of current density. The liquid junction potential, series resistance and leak current for these recordings were not corrected, and cells were discarded if series resistance was > 8 M Ω . The bath solution contained (in mM): NaCl 124, TEA-Cl 20, CaCl_2 2.0, MgCl_2 1, HEPES 5, glucose 10, pH 7.3 (adjusted with NaOH). The intracellular 10 Ca^{2+} solution contained (in mM): CsCl₂ 60, L-aspartic acid 80, 1,2-bis(o-amino phenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) 10, HEPES 10, pH 7.35 (adjusted with CsOH). The intracellular 10 Ca^{2+} solution contained (in mM): CsCl₂ 60, L-aspartic acid 80, BAPTA 1, CaCl_2 1, HEPES 10, pH 7.30

(adjusted with CsOH). Osmolarity was adjusted to 310 mOsm with sucrose for all solutions. The voltage-clamp protocols were generated using PatchMaster. Cells were voltage-clamped at a holding potential (V_h) of -120 mV, and currents were elicited by depolarizing pulses of 40 ms from -120 to $+45$ mV in 5 mV increments. Current density was calculated by normalizing to cell capacitance. Activation curves were obtained by transforming current data to conductance (G), which was calculated from the equation $G_{Na} = I/(V - E_{rev})$, where: I is the peak Na^+ current elicited by the depolarizing test potential; V is the test potential; and E_{rev} is the calculated Na^+ reversal potential. The ratio G/G_{max} was plotted against the membrane potential and fitted with the Boltzmann equation of the form: $G/G_{max} = 1/(1 + \exp((V - V_{1/2})/k))^{-1}$, where G_{max} is the extrapolated maximum conductance, V is the test voltage, $V_{1/2}$ is the half-activation voltage and k is the slope factor. Standard two-pulse protocols were used to generate the steady-state inactivation curves: from a holding potential of -120 mV, cells were stepped to 500-ms preconditioning potentials varying between -130 and -10 mV (prepulse) in 5 mV increments, followed by a 20-ms test pulse to -20 mV. Currents (I) were normalized to I_{max} and fit to a Boltzmann function of the form $I/I_{max} = 1/(1 + \exp((V_m - V_{1/2})/k))$ in which $V_{1/2}$ is the voltage at which half of $Na_v1.5$ channels are inactivated, k is the slope factor and V_m is the membrane potential. Data analysis was performed using FitMaster (HEKA Elektronik) and the Origin 8 software. Results are presented as means $\pm$ s.e.; the statistical significance of differences between groups was assessed using a two-tailed Student's t -test and was set at $P < 0.05$.

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Author contributions

C.W., S.-Y.L. and G.S.P. designed the study. C.W., B.C.C., H.Y. and H.-G.G. performed experiments. All authors analysed the data. S.-Y.L. and G.S.P. wrote the manuscript.

Disclaimer

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Additional information

Accession Numbers: Atomic coordinates and structure factors for the reported crystal structures have been deposited in the Protein Data Bank under accession codes 4JPZ and 4JQ0, for the $\text{Na}_v1.2/\text{Ca}^{2+}$ and $\text{Na}_v1.5/\text{Ca}^{2+}$ structures, respectively.

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