Ca$^{2+}$-dependent regulation of sodium channels Na$_{\text{V}}$1.4 and Na$_{\text{V}}$1.5 is controlled by the post-IQ motif

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Skeletal muscle voltage-gated Na$^+$ channel (Na$_{\text{V}}$1.4) activity is subject to calmodulin (CaM) mediated Ca$^{2+}$-dependent inactivation; no such inactivation is observed in the cardiac Na$^+$ channel (Na$_{\text{V}}$1.5). Taken together, the crystal structures of the Na$_{\text{V}}$1.4 C-terminal domain relevant complexes and thermodynamic binding data presented here provide a rationale for this isoform difference. A Ca$^{2+}$-dependent CaM N-lobe binding site previously identified in Na$_{\text{V}}$1.5 is not present in Na$_{\text{V}}$1.4 allowing the N-lobe to signal other regions of the Na$_{\text{V}}$1.4 channel. Consistent with this mechanism, removing this binding site in Na$_{\text{V}}$1.5 unveils robust Ca$^{2+}$-dependent inactivation in the previously insensitive isoform. These findings suggest that Ca$^{2+}$-dependent inactivation is effected by CaM's N-lobe binding outside the Na$_{\text{V}}$ C-terminal while CaM's C-lobe remains bound to the Na$_{\text{V}}$ C-terminal. As the N-lobe binding motif of Na$_{\text{V}}$1.5 is a mutational hotspot for inherited arrhythmias, the contributions of mutation-induced changes in CDI to arrhythmia generation is an intriguing possibility.
Voltage-gated Na\(^+\) channels (Na\(_V\)s) rapidly activate upon membrane depolarization to allow passage of Na\(^+\) ions into cells. Of the nine human isoforms of Na\(_V\), the two isoforms studied here are predominantly expressed in skeletal muscle (Na\(_V\)1.4) and cardiac tissue (Na\(_V\)1.5).

Structurally, Na\(_V\)s are large (~2000 residues) transmembrane proteins composed of four homologous domains (DI-DIV; each containing six transmembrane helices) that form a highly selective Na\(^+\) pore.\(^{2,3}\) DIV is followed by a C-terminal tail which contains a structural domain (CTerm, Na\(_V\)1.4 residues 1599–1754) consisting of a five-helix EF hand-like motif (EFh, helices αV–αV) followed by a long α-helix (helix αV1). helix αV1 contains an IQ (isoleucine-glutamine) motif ([ILV]Q–R----[R,K]), a high-affinity binding domain for calmodulin (CaM) in both its Ca\(^{2+}\)-free (apo) and Ca\(^{2+}\)-saturated form.\(^{3}\)

CaM is a small (148 residue) ubiquitous eukaryotic Ca\(^{2+}\)-sensing regulatory protein. CaM has two homologous lobes (N-lobe, helices A–D, residues 1–76; and C-lobe, helices E–H, residues 81–148) connected by a flexible linker; each lobe is capable of binding 2 Ca\(^{2+}\) ions cooperatively. Strong intra-lobe cooperativity means that CaM exists primarily in four states of Ca\(^{2+}\)-saturation: apo, (Ca\(^{2+}\)\(_2\))\(_N\).CaM, (Ca\(^{2+}\)\(_2\))\(_C\).CaM and (Ca\(^{2+}\)\(_3\))\(_C\).CaM. Each lobe may be closed, semi-open, or open depending on Ca\(^{2+}\)-saturation. A lobe in the semi-open or open state has a solvent-exposed hydrophobic cleft that may bind target peptides, such as the Na\(_V\) IQ motif, with high affinity.

Three crystal structures of Na\(_V\)1.5 CTerm/CaM complexes have been reported, each representing a distinct biological state: (1) Na\(_V\)1.5 CTerm in complex with apo CaM (PDB ID: 4OVN), (2) Na\(_V\)1.5 CTerm in complex with apoCaM plus fibroblast growth factor homologous factor (FFH; a Na\(_V\)-inactivating protein, PDB ID: 4DCK), and (3) Na\(_V\)1.5 CTerm in complex with (Ca\(^{2+}\)\(_2\))\(_C\).CaM and FFH (PDB ID: 4QJO). These structures provide snapshots of the interactions of Na\(_V\)1.5 CTerm and CaM as well as clues to possible CaM binding domains in Na\(_V\)1.4 CTerm; all show the C-lobe bound to the IQ-motif, with the N-lobe in varied configurations. Notably, structure 3 identifies a Ca\(^{2+}\)-N-lobe binding motif (NBLM) in helix αVI, i.e., a post-IQ motif located past where the C-lobe is bound.

Information regarding the global topology of Na\(_V\)s has been provided by three recent cryo-EM structures of eukaryotic voltage-gated sodium channels: cockroach NavPaS (P. americana, PDB ID: 5x0M; 37% identity to H. sapiens Na\(_V\)1.4),\(^{7}\) eel EnNa\(_V\) (E. electricus, PDB ID: 5XY6; 60% identity to H. sapiens Na\(_V\)1.4)\(^{8}\) and H. Sapiens Na\(_V\)1.4 (PDB ID: 6AGF).\(^{9}\) One cytosolic region, the linker between domain DIII and DVI (DIII–DIV linker), has already been implicated in channel regulation. In NavPaS the DIII–DIV linker passes between the CTerm of the channel and the membrane, while in the other two full Na\(_V\) structures the DIII–DIV linker is visible in a different orientation and no CTerm structure is observed. Previous studies have suggested that an IFM (Ile-Phe-Met) motif in the DIII–DIV linker is the putative fast inactivation gate. Studies have also suggested that the DIII–DIV linker interacts with CaM\(^{10,11}\) or Na\(_V\) CTerm\(^{12}\) and the possibility of DIII–DIV linker involvement in Na\(_V\) 1.4 Ca\(^{2+}\) regulation merits consideration.

Na\(_V\)1.4 and Na\(_V\)1.5 have distinct responses to increases in the Ca\(^{2+}\) concentration. Na\(_V\)1.4 exhibits a unique CaM mediated Ca\(^{2+}\)-dependent inactivation (CDI) which results in a reduction of bulk Na\(^+\) current by approximately 30% after the elevation of cellular Ca\(^{2+}\) levels to ~10 µM.\(^{13}\) In contrast, Na\(_V\)1.5 exhibits no CDI even at elevated Ca\(^{2+}\) levels. It is still not known what are the structural and thermodynamic mechanisms responsible for this difference in behavior are, given the high overall homology between the two Na\(_V\)s (65% identity, 1196 of 1836 residues), especially in the regulatory and CaM-binding CTerm (Na\(_V\)1.4 residues 1599–1754; 78% identity to Na\(_V\)1.5 CTerm).

Here, we present two crystal structures of the Na\(_V\)1.4 CTerm, one bound to apo CaM and the other bound to (Ca\(^{2+}\)\(_2\))\(_C\).CaM, as well as binding data between the CTerm of each isoform and CaM in four states of Ca\(^{2+}\)-saturation. Comparison of the two crystal structures reveals the Ca\(^{2+}\)-dependent changes experienced by CaM when bound to the Na\(_V\)1.4 CTerm. The binding data were used to model the populations of four CaM states bound to Na\(_V\)1.4 and 1.5 CTerm as a function of [Ca\(^{2+}\)] and [CaM]. In addition, electrophysiological data show that the CTerm of Na\(_V\) controls CDI and that deletion of the post-IQ NBLM in Na\(_V\)1.5 results in robust CDI. These structural, thermodynamic and electrophysiological data all support a mechanism of CDI in Na\(_V\)s controlled by the positioning of the N-lobe of CaM. Ca\(^{2+}\)-N-lobe binding to the post-IQ NBLM (WT of Na\(_V\)1.5) prevents CDI from occurring at elevated Ca\(^{2+}\) levels. The absence of a post-IQ NBLM sequence (Na\(_V\)1.4, Na\(_V\)1.5 with NBLM deleted) leads to CDI when the CaM N-lobe binds Ca\(^{2+}\). Thus, we showed that the structural and thermodynamic determinants of CDI reside in the CTerm and determine the physiological differences between the two Na\(_V\) isoforms’ response to Ca\(^{2+}\). These findings provide a path to identifying the possibility of Ca\(^{2+}\)-inactivation in other Na\(_V\) isoforms.

Results
Na\(_V\) C-terminal tail and CaM N-lobe control CDI. It was previously shown that Na\(_V\)1.4 responds to an increase in the Ca\(^{2+}\) concentration by reducing the maximum Na\(^+\) current by approximately 30% (calmodulin-dependent inactivation, CDI).\(^{13}\) This effect was observed in HEK293 cells expressing Na\(_V\)1.4 as well as in skeletal myotubes derived from mouse GLT cells. CDI was not observed in similar experiments involving expressed Na\(_V\)1.5. We performed electrophysiology experiments to confirm the Na\(_V\)-CTerm as the region responsible for the CDI as well as to determine the post-IQ region’s role in physiological Na\(_V\) function. The sequences of the two isoforms in the IQ and post-IQ regions are shown in Fig. 1f.

Electrophysiology experiments in HEK293 cells were performed using a chimera in which the C-terminal tail of Na\(_V\)1.5 (aa 1774–2016) was replaced by its Na\(_V\)1.4 counterpart (aa 1599–1836). Ca\(^{2+}\) was delivered using voltage pulses that selectively activate voltage-gated Ca\(^{2+}\)-channel Ca\(_V\)2.1 which was co-expressed with the Na\(_V\) channels. The chimeric protein (Na\(_V\)1.5-CTa11.4; Na\(_V\)1.4–1773 with Na\(_V\)1.4 1599–1836) shows CDI, in presence of CaM (Fig. 1a–d). The opposite experiment, Na\(_V\)1.4-CTa11.5, was reported previously and does not show CDI.\(^{13}\) The participation of CaM as the Ca\(^{2+}\) sensor for CDI was confirmed using two engineered CaM mutants with Ca\(^{2+}\) binding sites binding disabled either in the N or the C lobe: CaM2 (D20A, D56A; N lobe Ca\(^{2+}\) binding disabled) or CaM3 (D93A, D129A; C lobe Ca\(^{2+}\) binding disabled). Only WT CaM and CaM3 were capable of inducing CDI (Fig. 1e). These experiments show that CDI is dependent on the Na\(_V\) C-terminal tail and CaM. Furthermore, the C-lobe of CaM is not required to bind Ca\(^{2+}\); the N-lobe binding Ca\(^{2+}\) is sufficient to cause CDI.

Crystal structures of the Na\(_V\)1.4 CTerm–CaM complexes. The structure of the Na\(_V\)1.4 CTerm–CaM complex was determined in the absence and in the presence of 10 mM Ca\(^{2+}\). Extensive crystallization trials using two different Na\(_V\)1.4 CTerm constructs (Long (residues 1599–1764) and Short (residues 1599–1754)—produced the crystals used in the structure determinations.

Crystal structure of Na\(_V\)1.4 CTerm bound to apoCaM. The 1.8 Å resolution structure of Na\(_V\)1.4 CTerm in complex with apo CaM (R\(_{work}/R_{free}\) = 20.3/23.4) contains one complex in.
the asymmetric unit (ASU) of the cell (Fig. 2a, Table 1, Supplementary Fig. 1). The NaV1.4 CTerm contains the 5-helix globular EFL (αI–αV, residues Glu1614–Met1706; residues 1599–1613 not observed) followed by a long helix (αVI, residues Leu1722–Lys1748). Helix αVI is in the same orientation relative to the EFL as seen in NaV1.5 CTerm in complex with apo CaM (PDB ID: 4OVN)4. In contrast, there is a rotation of ~90° of helix αVI relative to the EFL in the structure of NaV1.5 CTerm, apo CaM and FHF (PDB ID: 4DCK)5. The apo CaM C-lobe is in a semi-open configuration, and bound to the IQ-motif of helix αVI with a buried surface area (BSA) of 872 Å² (Fig. 2b)14. The apo C-lobe contacts appear to be anchored by two NaV residues which are in the C-lobe's hydrophobic pocket: Ile1734 (120 Å² BSA) and Tyr1738 (119 Å² BSA). The CaM C-lobe also forms four Glu–Arg

NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-019-09570-7 | www.nature.com/naturecommunications
Fig. 1 C-terminal tail and CaM control of Na⁺ CDI. a Schematic of Naᵥ1.5, Naᵥ1.4 and Naᵥ1.5-CTail1.4 (Naᵥ1.5 residues 1–1773 with Naᵥ1.4 1599–1836) used in experiments below. b Pulse protocol for Na⁺ channel current recordings and assessment of CDI. The pulse protocol with a 150 msec step to +20 mV (red) activates co-expressed Caᵥ2.1 while a step to −40 mV (black) does not. c Current elicited by the pulse protocols. The Na⁺ current is probed before (P1) and after (P2) Ca²⁺ entry into the cells due to the intermediate depolarization at −40 mV (where the Ca²⁺ channels are closed) and +20 mV (where the Ca²⁺ channels are open). The pre-pulse P1 and the test pulse P2 are used to probe the Na⁺ current in the absence of Ca²⁺ due to the intermediate depolarization at −40 mV or in the presence of Ca²⁺ due to the activated Ca²⁺ channels during the further depolarization at +20 mV. d Na⁺ currents measured during P1 and P2. The P2 current after Ca²⁺ influx (red trace) compared to P2 current with no Ca²⁺ influx (black). e Data points of CDI measurement (open circles, filled circle as mean with bars showing ±1 standard deviation) with CaM WT, CaM12 or CaM34 showing only the N-lobe of CaM is required to bind Ca²⁺ for CDI to occur. Statistical significance was determined by an unpaired t-test. Supplementary Table 2 lists values of the individual data points shown. f Alignment of the sequences of Naᵥ1.4 and Naᵥ1.5 in IQ and post-IQ regions.

Fig. 2 Structures of Naᵥ1.4 CTerm in complex with CaM, ± Ca²⁺. a 1.8 Å resolution structure of the complex of the Naᵥ1.4 CTerm and apo CaM. CTerm colored gray and CaM light teal (N-lobe) and light orange (C-lobe). b Close-up of the apo C-lobe and helix α VI interactions. c Close-up of N-lobe and EFL interaction. d 3.3 Å resolution structure of the complex of the Naᵥ1.4 CTerm and (Ca²⁺)₄-CaM. CTerm is colored black and CaM dark teal (N-lobe) and dark orange (C-lobe). e Close-up of Ca²⁺-C-lobe and helix αVI interactions. f Close-up of Ca²⁺-N-lobe and EFL interaction. Residues shown have more than 10 Å² BSA. g–i Schematics of CaM N-lobe control in Naᵥ regulation. g Activated conformation of CaM and Naᵥ1.4 (PDB ID: 6MBA). h Ca²⁺-inactivated conformation of CaM and Naᵥ1.4 (PDB ID: 6MC9). i Ca²⁺-insensitive conformation of CaM and Naᵥ1.5 (PDB ID: 4JQ0, FHF molecule not displayed).
The absence of Ca\(^{2+}\) burying 495 Å\(^2\) (Fig. 2c). The apo N-lobe contacts are anchored by Glu11, which is involved in salt-bridges with NaV1.4 CTerm: one with NaV residues Ile1659 (83 Å\(^2\) BSA) and Leu1722 (72 Å\(^2\) BSA). Unlike in the complex with the apo CaM, the C-lobe does not form any salt bridges with the NaV1.4 CTerm (Supplementary Fig. 2). The NaV1.4 CTerm shows the same fold as NaV1.5 EFL6,17.

Crystal structure of NaV1.4 CTerm bound to (Ca\(^{2+}\))\(_{4}\)–CaM. The crystal structure of NaV1.4 CTerm Short (residues 1599–1754) in complex with (Ca\(^{2+}\))\(_{4}\)–CaM was determined to a resolution of 3.30 Å (R\(_{work}\)/R\(_{free}\) = 24.0/28.5). Crystals belong to the space group P4\(_3\)212 and contain one complex in the ASU with four Ca\(^{2+}\) ions. Anomalous scattering was used to confirm the presence of bound Ca\(^{2+}\) ions. The same crystal used for structure determination of NaV1.4 CTerm bound to (Ca\(^{2+}\))\(_{4}\)–CaM was used to collect data at a wavelength of 2.515 Å, near the anomalous edge of Ca\(^{2+}\) (3.070 Å) (Table 1). These data were used to calculate an anomalous map at 3.9 Å resolution. This map showed five peaks above 4σ above 4σ contour of the map corresponding to the positions of the 4 Ca\(^{2+}\) ions, one in each EF-hand loop of CaM (Fig. 3c,d), as well as the sulfur of Met 1668 in the CTerm. The anomalous scattering signal confirms the presence of Ca\(^{2+}\)-with high occupancy in CaM in the crystal and also eliminates the possibility of Ca\(^{2+}\)-binding sites within the NaV1.4 EFL itself, in agreement with previous reports of weak or no-binding between Ca\(^{2+}\) and NaV1.5 EFL.6,17.

### Table 1 Data collection and refinement statistics (molecular replacement)

| Na\(_{\alpha1.4}\) CTerm + apoCaM PDB: 6MBA | Na\(_{\alpha1.4}\) CTerm + (Ca\(^{2+}\))\(_{4}\)–CaM PDB: 6MC9 | Na\(_{\alpha1.4}\) CTerm + (Ca\(^{2+}\))\(_{4}\)–CaM Anomalous Data |
|-----------------------------------------|-------------------------------------------------|--------------------------------------------------|
| **Data collection**                     |                                                 |                                                  |
| Cell dimensions \(a, b, c (Å)\)         | 112.7, 29.0, 95.0                               | 90.0, 123.6, 90.0, 90.0                           |
| Resolution (Å)                          | 44.87–179                                       | 186–179                                         |
| \(R_{merge}\)                            | 0.072 (0.456)                                   | 0.050 (0.312)                                   |
| Completeness (%)                         | 98.0 (96.4)                                     | 98.0 (96.4)                                     |
| Wavelength (Å)                           | 0.918                                           | 0.918                                           |
| Refinement                               | 23.880                                          | 20.230/0.234                                   |
| No. reflections                         | 5840                                            | 0.240/0.285                                    |
| R\(_{work}\)/R\(_{free}\)               | 2.128                                           | 2.126                                           |
| No. atoms                                | 20                                              | 120                                             |
| Protein                                 | 56                                              | 56                                              |
| Ligand/ion                              | 55                                              | 95                                              |
| Water                                   | 47                                              | NA                                              |
| R.m.s. deviations                       | 0.157                                           | 0.101                                           |
| Bond lengths (Å)                        | 0.004                                           | 0.008                                           |
| Bond angles (°)                         | 1.53                                            | 1.06                                            |

Values in parentheses are for highest-resolution shell.

The Ca\(^{2+}\)-saturated CaM N-lobe conformation differs significantly from the one seen in the apo–CaM complex (Fig. 2f). The N-lobe’s hydrophobic cleft, formed between helices A–E, is now open and solvent exposed but despite having a 12-residue DIII-DIV linker peptide in the crystallization media, no peptide is observed bound inside the cleft. In contrast, no large conformational change is observed in the CaM C-lobe bound to the NaV1.4 CTerm upon Ca\(^{2+}\) addition (Fig. 3b and Supplementary Fig. 2). Anomalous scattering was used to confirm the presence of bound Ca\(^{2+}\) ions. The same crystal used for structure determination of NaV1.4 CTerm bound to (Ca\(^{2+}\))\(_{4}\)–CaM was used to collect data at a wavelength of 2.515 Å, near the anomalous edge of Ca\(^{2+}\) (3.070 Å) (Table 1). These data were used to calculate an anomalous map at 3.9 Å resolution. This map showed five peaks above 4σ contour of the map corresponding to the positions of the 4 Ca\(^{2+}\) ions, one in each EF-hand loop of CaM (Fig. 3c,d), as well as the sulfur of Met 1668 in the CTerm. This strong anomalous scattering signal confirms the presence of Ca\(^{2+}\)-with high occupancy in CaM in the crystal and also eliminates the possibility of Ca\(^{2+}\)-binding sites within the NaV1.4 EFL itself, in agreement with previous reports of weak or no-binding between Ca\(^{2+}\) and NaV1.5 EFL.6,17.

Inspection of crystal packing in the NaV1.4 CTerm and (Ca\(^{2+}\))\(_{4}\)–CaM crystal shows an intermolecular interaction between the CTerms of symmetry-related molecules: the end of helix αVI interacts with a groove in the EFL of a neighboring CTerm (Supplementary Fig. 3). The EFL’s groove is formed by helices αI, IV, and V with 841 Å BSA. No salt bridge or hydrogen bond interactions are formed in the CTerm and helix αVI interaction and the complex formation significance score (CSS) is...
0.00, which strongly suggests that this interaction is a result of crystal packing \(^1\). It is possible that this interaction is mimicking physiological DIII–DIV linker and EFL interactions (see Discussion).

**Binding measurements of Nav\(_x\) CTerm to CaM.** The binding affinities of Nav\(_{1.4}\) and Nav\(_{1.5}\) CTerm for CaM were determined using isothermal titration calorimetry (ITC). Titrations were conducted under both Ca\(^{2+}\)-free (with 50 \(\mu\)M EGTA) and Ca\(^{2+}\)-saturating conditions (with 1 mM CaCl\(_2\)). As CaM has two pairs of EF-hands, Ca\(^{2+}\)-occupancy of CaM sites is more complex than the binary apo or fully Ca\(^{2+}\)-saturated captured in these two crystal structures. Each CaM EF hand is capable of binding one Ca\(^{2+}\) ion so there are 16 possible states of Ca\(^{2+}\)-saturation. However, because of the cooperativity between Ca\(^{2+}\) binding to the two EF hands in each lobe (i.e., between EF hands 1 and 2 in the N-lobe and between EF hands 3 and 4 in the C-lobe), states in which each lobe is either empty or has 2 Ca\(^{2+}\) ions will be the most highly populated. These considerations lead to an approximate model with CaM in 4 states of Ca\(^{2+}\)-saturation: apo, (Ca\(^{2+}\))\(_2\)_C-CaM, (Ca\(^{2+}\))\(_2\)_N-CaM and (Ca\(^{2+}\))\(_4\)-CaM. Accordingly, binding measurements were performed using CaM\(_{12}\) or CaM\(_{34}\) under high calcium conditions to capture the CTerm binding affinity for (Ca\(^{2+}\))\(_2\)_C-CaM and (Ca\(^{2+}\))\(_2\)_N-CaM, respectively. In total, these binding experiments provided data with CaM’s four most physiologically relevant states. Both lengths of the CTerm used in the structural studies were used in the experiments: Long (residues 1599–1764) and Short (residues 1599–1754). Long is considered to reflect physiological binding more accurately and was used in the thermodynamic modeling; Short crystallized with (Ca\(^{2+}\))\(_4\)-CaM and Short CTerm binding measurements are also reported here (Supplementary Figs. 4–7).

Binding affinities are presented in Table 2. Control titrations using apo CaM\(_{12}\) and CaM\(_{34}\) were also performed, confirming their function as mimics of half-saturated WT CaM (Supplementary Fig. 8, Supplementary Table 1).

The \(K_s\) of CaM binding to the skeletal muscle Nav\(_{1.4}\) CTerm Long are tighter than 1 \(\mu\)M for all conditions tested (Table 2). Apo CaM displays the highest affinity (\(K_d = 17\) nM). The intermediate Ca\(^{2+}\)-saturated states show similar affinity [(Ca\(^{2+}\))\(_2\)_C-CaM \(K_d = 154\) nM, (Ca\(^{2+}\))\(_2\)_N-CaM \(K_d = 121\) nM]. (Ca\(^{2+}\))\(_4\)-CaM shows the weakest binding (\(K_d = 275\) nM) indicating a general trend: the more Ca\(^{2+}\)-saturated the species of CaM the more they are (modestly) penalized for NaV1.4 CTerm binding.

The \(K_s\) of CaM binding to the cardiac NaV1.5 CTerm Long range from 48 to 90 nM for three of the species: apoCaM, (Ca\(^{2+}\))\(_2\)_N-CaM, and (Ca\(^{2+}\))\(_4\)-CaM (Table 2). The fourth CaM species, (Ca\(^{2+}\))\(_2\)_C-CaM, binds the NaV1.5 CTerm with a \(K_d\) of 3.6 \(\mu\)M, or about 40-fold weaker binding than any other CaM species. The dramatically weakened binding of (Ca\(^{2+}\))\(_2\)_C-CaM to NaV1.5 CTerm contrasts with the trend observed in Nav\(_{1.4}\), for which (Ca\(^{2+}\))\(_2\)_C-CaM binds with high affinity to the CTerm. For the NaV1.5 CTerm the two tightest binding CaM species are the two physiological endpoint species: apo CaM (\(K_d = 48\) nM) and fully saturated (Ca\(^{2+}\))\(_4\)-CaM (\(K_d = 70\) nM) which is a trend captured in our thermodynamic model.

**Thermodynamic analysis of CTerm and CaM binding data.** The differences in affinity of the two isoforms’ CTerm for the four CaM species must result in different species being present at each Ca\(^{2+}\) concentrations and determine the different physiological responses to elevated Ca\(^{2+}\) concentration. We employed a thermodynamic model that provides estimates of the populations of Nav\(_x\) CTerm and CaM species at varying [Ca\(^{2+}\)] and [CaM]. To start, the fractions of the CaM species as a function of [Ca\(^{2+}\)] can be calculated using the previously reported binding data, which describes Ca\(^{2+}\) affinities for the N- and C-lobe of full-length mammalian CaM\(_{18,19}\) (Fig. 4a).

These affinities provide the necessary information to compute the fraction \(f_X\) of all four species as a function of [Ca\(^{2+}\)] (Supplementary Fig. 9).

Since the total number of Na\(_V\) channels is much smaller than the number of CaM molecules, the CaM populations in Fig. 4a will not be affected by binding to the Nav\(_x\) CTerm. In this case, the fractions of five CTerm-CaM species (including free CTerm) can be calculated as a function of the CaM species, using the binding constants reported here (Table 2) and the equations presented in Fig. 4a, b.

This analysis links the concentrations of CaM species, and their [Ca\(^{2+}\)] dependence, to the species in Fig. 4b. By estimating the fractional population of Nav\(_x\) CTerm species we sidestep working with the concentration of transmembrane Na\(_V\). This is particularly useful as the 2-D concentration of membrane-bound species is difficult to measure experimentally and the theoretical framework for thermodynamic analysis of 2-D species is less developed. Our model allows the resulting CaM/Ca\(^{2+}\)/CTerm species populations to be readily calculated from relevant experimental variables and soluble species: [CaM] and [Ca\(^{2+}\)] (Fig. 4c–f). The Ca\(^{2+}\)-dependent trends for each isoform are less sensitive to the

| Sample Cell | [Ca\(^{2+}\)] nM | Titrant | \(K_d\) nM | \(\Delta G\) Kcal mol\(^{-1}\) |
|-------------|----------------|--------|---------|------------------|
| Nav1.4 CTerm Long | 0 | apoCaM | 17 ± 3 | 10.59 |
| Nav1.4 CTerm Long | 1 | (Ca\(^{2+}\))\(_4\)-CaM | 275 ± 26 | 8.94 |
| Nav1.4 CTerm Long | 1 | (Ca\(^{2+}\))\(_2\)_C-CaM | 154 ± 9 | 9.29 |
| Nav1.4 CTerm Long | 1 | (Ca\(^{2+}\))\(_2\)_N-CaM | 121 ± 7 | 9.43 |
| Nav1.5 CTerm Long | 1 | apoCaM | 48 ± 5 | 9.98 |
| Nav1.5 CTerm Long | 1 | (Ca\(^{2+}\))\(_2\)_C-CaM | 89 ± 7 | 9.61 |
| Nav1.5 CTerm Long | 1 | (Ca\(^{2+}\))\(_2\)_N-CaM | 360 ± 200 | 7.43 |
| Nav1.5 CTerm Long | 1 | (Ca\(^{2+}\))\(_3\)_N-CaM | 90 ± 4 | 9.58 |
| Nav1.4 CTerm Short | 0 | apoCaM | 114 ± 11 | 9.47 |
| Nav1.4 CTerm Short | 1 | (Ca\(^{2+}\))\(_3\)_N-CaM | 305 ± 21 | 8.88 |
| Nav1.4 CTerm Short | 1 | (Ca\(^{2+}\))\(_2\)_C-CaM | 156 ± 5 | 9.28 |
| Nav1.4 CTerm Short | 1 | (Ca\(^{2+}\))\(_2\)_N-CaM | 282 ± 6 | 8.93 |
| Nav1.5 CTerm Short | 0 | apoCaM | 39 ± 3 | 10.10 |
| Nav1.5 CTerm Short | 1 | (Ca\(^{2+}\))\(_2\)_C-CaM | 1520 ± 90 | 7.93 |
| Nav1.5 CTerm Short | 1 | (Ca\(^{2+}\))\(_2\)_N-CaM | 5260 ± 240 | 7.20 |
| Nav1.5 CTerm Short | 1 | (Ca\(^{2+}\))\(_3\)_N-CaM | 376 ± 12 | 8.76 |
Fig. 4  

**NaV1.4 CTerm and NaV1.5 CTerm populations with bound CaM.**  

**a** Reaction scheme of Ca$^{2+}$ ions binding to CaM lobes. Thermodynamic data of these binding reactions was reported previously.  

**b** Reaction scheme of CaM in four Ca$^{2+}$-saturation states binding to NaV CTerm. Thermodynamic data of these binding reactions are reported here.  

**c–f** Panels showing the relative population (Z-axis) of four CaM species bound to NaV CTerm, modeled as a function of [Ca$^{2+}$] and [CaM] using the two schemes above. NaV1.4 CTerm-bound species on the left, NaV1.5 CTerm-bound CaM-species on the right.  

**c** Population of apo CaM bound to NaV CTerm.  

**d** Population of (Ca$^{2+}$)$_2$-CaM bound to NaV CTerm.  

**e** Population of (Ca$^{2+}$)$_2$-N-CaM bound to NaV CTerm.  

**f** Population of (Ca$^{2+}$)$_4$-CaM bound to NaV CTerm. Only NaV1.4 CTerm shows a significant population of bound (Ca$^{2+}$)$_2$-C-CaM (orange surface).  

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**CaM concentration and are easiest to visualize at constant [CaM] (Fig. 5, Supplementary Fig 10).**  

**The post-IQ region of NaV** controls CDI response. To identify specific regions of the CTerm contributing to the CDI we analyzed the available NaV1.5 CTerm and CaM structures. The structure of NaV1.5 CTerm in complex with (Ca$^{2+}$)$_4$-CaM and FHFP (PDB ID: 4JQ0) identified the post-IQ NLBM as residues 1916–1927 (LLQRSLKHASFL) with the N-lobe binding burying two NaV residues in its hydrophobic cleft: Leu1917 and Leu1921. In contrast, the structures and binding model presented here suggest NaV1.4 CTerm lacks such an NLBM. Accordingly, a C-terminally truncated NaV1.5 with the post-IQ NLBM removed (NaV1.5ΔP-IQ, last residue Ser1920) was used in electrophysiology experiments. This truncation, in the middle of the Ca$^{2+}$-N-lobe binding domain, eliminates the 2nd anchor Leu and any other subsequent downstream NaV-CaM contacts. Photo-uncaging Ca$^{2+}$ in HEK293 cells expressing the NaV1.5 ΔP-IQ channel leads to robust CDI not observed in full-length NaV1.5 (Fig. 6). These experiments are consistent with the NaV1.5 post-IQ NLBM preventing the development of CDI in this channel.  

**Discussion**  

NaV1.4 exhibits robust regulation by CaM: apo CaM bound to the channel’s CTerm causes an increase in channel open probability, whereas elevated Ca$^{2+}$ (~10 µM) causes the rapid onset of CaM-mediated CDI. In contrast, NaV1.5 which also binds CaM in its CTerm, does not exhibit CDI at elevated Ca$^{2+}$ levels. This isoform-specific response has been shown to be CTerm controlled and to require bound CaM. The strongest evidence for the localization of this effect is shown in Fig. 1: a chimera containing the NaV1.5 four transmembrane domains followed by the NaV1.4 C-terminal tail exhibits robust CDI. Understanding the different responses of the two isoforms to the increase in the [Ca$^{2+}$] requires the detailed identification of the structural determinants of the CDI as well as the thermodynamics of binding between the two CTerm regions and CaM at different Ca$^{2+}$ concentrations. We carried out binding and structural studies to characterize the interactions of CaM with the NaV1.4 CTerm. Apo CaM shows high affinity for NaV1.4 CTerm Long ($K_d = 17$ nM, Table 2), indicating that CaM is likely bound to the CTerm of NaV at resting Ca$^{2+}$ levels. The structure of apo CaM bound to the NaV1.4 CTerm, determined to 1.8 Å resolution, shows that the CaM C-lobe binds to the NaV IQ motif in helix αVI in a semi-open conformation and that the CaM N-lobe makes surface contacts with the CTerm EFL (Fig. 2a–c). This structure is highly similar to that of the complex of NaV1.5 CTerm and apo CaM (PDB ID: 4OVN, rmsd 2.71 Å, chains D and I). The similarities between the NaV1.4 and NaV1.5 CTerm complexes with apo CaM, in both conformation and binding affinities (NaV1.5-CTerm Long $K_d = 48$ nM, Table 2) and the equivalence of the response of both isoforms to apo CaM binding (i.e., increased activation) suggest that the conformation of the
CTerm does not show as in NaV1.5 CTerm has primarily (Ca2+)-sodium channel NaV1.5 displaying CTerm and post-IQ NLBM region; I and after uncaging is shaded. Current measurements of NaV1.5 peak currents is outlined in red and the difference in peak current before IQ deletion; Na before uncaging. Bottom, mean ± ecm for 8 NaV1.4-apo CaM crystallized here corresponds to an activating conformation of apo CaM bound to the NaV1.4 CTerm.

Deletion of NaV1.5 post-IQ NLBM reveals CDI. a Schematic of the sodium channel NaV1.5 displaying CTerm and post-IQ NLBM region; currents unaffected by 10 µM Ca2+. Gray dots, peak currents before uncaging. Bottom, mean ± ecm for CDI versus Ca2+-step amplitude. CDI = 1 –average peak Ipeak of last three to four responses after Ca2+ uncaging/peak Ipeak before uncaging. b Schematic of NaV1.5 displaying CTerm and the post-IQ deletion; Na+ currents reduced strongly at 10 µM Ca2+, the envelope of peak currents is outlined in red and the difference in peak current before and after uncaging is shaded. Current measurements of NaV1.5ΔP-IQ, shown as in (a).

NaV1.4-apo CaM crystallized here corresponds to an activating conformation of apo CaM bound to the NaV1.4 CTerm.

(Ca2+)4−CaM binds with high affinity to the NaV1.4 CTerm (Table 2), indicating that CaM remains bound to the CTerm at elevated Ca2+. The crystal structure of (Ca2+)4−CaM in complex with NaV1.4 CTerm Short shows that the C-lobe of (Ca2+)4−CaM binds the IQ motif of helix αVI, in a pose similar to that seen in the apo CaM CTerm complex (Fig. 2a, b). In this complex Ca2+ binding does not open the C-lobe’s hydrophobic cleft; instead, the C-lobe is fully Ca2+-saturated and yet maintains a semi-open configuration (Fig. 3b, d; Supplementary Fig. 2). The structure reported here suggests that the C-lobe serves to anchor CaM to the NaV1.4 IQ motif, both in the presence and absence of Ca2+, maintaining the same conformation and the same interface.

The N-lobe of Ca2+-CaM is found in an open configuration, in contact with the Na, EFL, with a shifted center of mass (3.6 Å shift) and with a reduced BSA relative to the apo CaM N-lobe EFL interface (334 vs. 495 Å2). The hydrophobic cleft of the Ca2+-saturated N-lobe is open, solvent exposed and unoccupied in this structure (Fig. 3a). Even though the NaV1.4 CTerm construct used here includes the entire post-IQ NLBM previously identified in NaV1.5 CTerm (NaV1.4 residues 1742–1753, LLQRSMKQASYM, 67% identity to NaV1.5), no N-lobe binding post-IQ was observed here. This difference indicates that the post-IQ region is crucial for control of Ca2+-CaM N-lobe binding.

The largest difference between the affinities of CaM for the NaV1.4 and 1.5 CTerm is found in the species with Ca2+ only in the C-lobe (Table 2). The (Ca2+)2−CaM12 species binds NaV1.4 CTerm Long with a Kd = 154 nM and NaV1.5 CTerm Long with a Kd = 3.6 µM. With CTerm Short the situation is similar: (Ca2+)2−CaM12 has a Kd = 156 nM for NaV1.4 and Kd = 5.26 µM for NaV1.5. This large difference may, in part, be responsible for the differences in CDI between the isoforms. Nevertheless, a complete explanation of the differences requires an analysis of all the species contributing to the control of the channel’s activity. To this effect, the binding measurements were used to model the populations of NaV CTerm-CaM species as a function of [CaM] and [Ca2+]. Using the experimental affinities, this model provides the relative populations of the five CTerm species in Fig. 2 as a function of [Ca2+] and [CaM] that simulate rapid Ca2+ signaling in a cell, or the variation of CaM concentration across cells or experiments (Fig. 4).

The populations obtained with this model show similar trends for bound-CaM behavior with both NaV isoforms at the extremes of low or high Ca2+ concentration. These trends are most defined at [CaM] of ~10 µM which is near the measured concentration of...
CaM in cardiac myocytes and skeletal muscle cells (Fig. 5a). For both isoforms, apo CaM bound to NaV CTerm is the dominant species at physiological resting Ca2+ levels (~100 nM) while (Ca2+)2–CaM bound to NaV CTerm is the dominant species at high Ca2+ levels (~100 µM). The isoform populations are most divergent at 10 µM Ca2+ at which concentration NaV1.4 has a high population of half Ca2+-saturated CaM, (Ca2+)2–CaM, while NaV1.5 is switching from bound apo CaM to bound (Ca2+)2–CaM with nearly absent intermediate populations.

This isoform-specific tuning of CaM behavior occurs at a Ca2+ concentration that corresponds to those found in relevant cell Ca2+ signaling events and which leads to CDI. The half Ca2+ saturated species of CaM, (Ca2+)2–CaM, predicted bound to NaV1.4 CTerm may then be essential to the molecular mechanism of CDI. However, inspection of the crystal structures presented here shows that Ca2+ binding to the C-lobe does not have a major effect on its conformation nor CaM interaction with the NaV1.4 CTerm. Instead, it is the lack of Ca2+ binding by the N-lobe when the CaM C-lobe is bound to NaV1.4 CTerm, at 10 µM Ca2+ that is mechanistically relevant. The affinity of Ca2+ for the CaM N-lobe is intrinsically low (Kd = 20 µM) and Ca2+ binding is enhanced when bound to a target helix. This lack of Ca2+ binding is consistent with a mechanism in which the NaV1.4 CTerm lacks a functional NLBM and the N-lobe is free to bind another region of the channel. In contrast, with the NaV1.5 CTerm, which does contain the NLBM, the Ca2+-N-lobe binds to this motif and is unavailable. This is supported by the thermodynamic binding model which shows that the N-lobe of CaM binds to NaV1.5 CTerm more readily when it binds Ca2+ (Fig. 5b). If the Ca2+-N-lobe does not bind post-IO it is free to bind another cytoplasmic region of the NaV. This is the interaction that results in CDI. One suggested target for CaM binding in NaV regulation is the cytosolic DIII–DIV linker, (residues 1292–1354 in NaV1.4)34. In a recent Na+ channel structure, NavPaS (PDB ID: 5x0M)5 as well as in the CaV1.1 structure (PDB ID: 5GJV)11, a portion of the DIII–DIV linker makes contact with the EFL in a pocket formed by helices αI, IV, and V, suggesting the DIII–DIV linker would be near to bound CaM in NaV1.4 (Supplementary Fig. 11). Two previous structures, the NaV1.5 residues Q1491–1501L bound to the Ca2+-saturated C-lobe (PDB ID: 4DJ0)11 and NaV1.5 residues L1514–1522K bound to Ca2+-saturated N-lobe (PDB ID: 5DBR)29, show helical portions of the NaV1.5 DIII–DIV linker (NaV1.4 residues 1467–1529) bound to (Ca2+)2–CaM.

We tested whether the Ca2+-N-lobe of CaM attached to the NaV1.4 CTerm by the Ca2+-C-lobe binding to the IQ motif can bind the DIII–DIV linker. However, attempts at co-crystallizing a peptide that spans residues 1316–1327 of the DIII–DIV linker (first helix) and the NaV1.4 CTerm (Ca2+)2–CaM–complex produced crystals but showed no density that could be attributed to the peptide. Furthermore, ITC experiments titrating the NaV1.4 CTerm and (Ca2+)4–CaM complex with two DIII–DIV linker peptides (12 or 20 residues) exhibit no binding even at high peptide concentrations (Supplementary Fig. 12). Binding to the DIII–DIV linker, however, cannot be fully ruled out. Binding as a free peptide vis a vis the physiological situation has to overcome two large endogenic processes: (1) stabilization of the helix as required for binding, since the free peptide may not be helical in solution and (2) bringing the peptide and the binder together that in the physiological situation might already be proximal as part of the same complex (chelate effect). Independently of which region is recognized, a proximal binding partner in the NaV1.4 for the CaM–Ca2+ N-lobe brings together the known crystal structures, the thermodynamic model and the existing physiological data.

The strongest evidence that NaV CDI is controlled via a distal binding partner of the Ca2+-N-lobe is provided by electrophysiology experiments using NaV1.5. Unlike NaV1.4, WT NaV1.5 is insensitive to Ca2+ concentrations of up to 10 µM and shows no CDI (Fig. 6a). Here we showed that robust CDI may be revealed in NaV1.5 by a deletion of the post-IQ NLBM (Fig. 6b); that is, elimination of the NLBM results in CDI.

Our results, along with other previously published data, strongly suggest a molecular mechanism of CDI in NaV in which the CTerm and CaM interactions play a crucial role. CaM is anchored by its C-lobe binding to the IQ motif of the NaV CTerm and this interaction is independent of [Ca2+]2. The N-lobe of CaM acts as a Ca2+–dependent switch: it is closed and makes surface contacts with the EFL under apo conditions; upon binding Ca2+ the N-lobe switches to the open conformation. The N-lobe is then poised to bind a nearby target in the cytoplasmic regions of the channel. In NaV1.5 the Ca2+-N-lobe binds the post-IQ NLBM and no CDI results (Supplementary Fig. 13). In NaV1.4 the N-lobe has a much lower affinity for the post-IQ NLBM and binding to a another N-lobe partner results in CDI. Differences in the post-IQ motif between NaV1.4 and NaV1.5 are likely the cause of this isoform-specific behavior in Ca2+-N-lobe binding. Several residues differ between the isoforms in the post-IQ motif (Fig. 1, Supplementary Fig. 14) including the change of Leu1921 of NaV1.5 to Met (1747) in NaV1.4. Leu1921 is buried in the N-lobe’s hydrophobic pocket and makes a close contact with Met37 in CaV1.2–CaM (PDB ID: 4JQ0). The change of Leu to Met combined with other differences may lead to steric clashes, resulting in a weaker affinity of the Ca2+-N-lobe for the post-IQ motif of NaV1.4. This proposal identifies a motif that can be used to facilitate the study of Ca2+-regulation in other NaV isoforms. Isoform-specific CDI may represent an additional level of regulation of NaV channels reflecting the different characteristics of their currents: periodicity (or not), frequency and duration.

Also, as the post-IQ is a mutational hotspot for inherited arrhythmias, the contributions of mutation-induced changes in CDI to arrhythmia generation is an intriguing possibility26,27. The same effects may be operational in neuronal isoforms that mimic the behavior of NaV1.5 leading to neurological disorders such as seizures or neuropathic pain.

Methods

Human NaV1.5–CaTl1.4 chimera electrophysiology. The H. sapiens NaV1.5–eGFP fusion construct was cloned by inserting the coding region of NaV1.5 into EGF-P-N3 (Clontech)26. A chimera was created with NaV1.5 up to amino acid 1273 followed by NaV1.4 C-terminal tail (residues 1599–1836) and enhanced green fluorescent protein (NaV1.5–CaTl1.4). Approximately 0.75 × 106 HEK293 cells (American Type Culture Collection, Manassas, VA) were cultured in 6-well tissue culture dishes in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM/L), penicillin (100 U/mL), streptomycin (10 mg/mL) and gentamicin (50 mg/mL). The cells were co-transfected with plasmids encoding the appropriate NaV1.5–CaTl1.4, CaM, Ca2+ EFb 43/44+/44+/47+/48+/49+ and β1/2 α subunits at 1 µg/mL29,30. Cells were transfected using LipoFectamineTM 2000 (Invitrogen) according to the manufacturer’s instructions and were studied 48 to 72 h post-transfection. The total amount of DNA for all transfections was kept constant.

The bath solution contained (in mM): NaCl 130; CaCl2 15; MgCl2 1; KCl 4; Na2HPO4 0.33; HEPS, 10; with pH 7.4 adjusted with NaOH and at 290 mOsm adjusted with NaCl. The pipet solution, “0.5 EGTA” contained (in mM): CaMeSO4 12; CsCl 5; MgCl2 1; MgATP, 4; HEPS (pH 7.4), 10; and EGTA 0.5; at 290 mOsm adjusted with glucose.

Whole cell IiNa and Ic were recorded under voltage-clamp with an Axopatch 200 A patch-clamp amplifier (Molecular Devices Corp., Sunnyvale, CA) at room temperature (22 °C). Voltage command protocols were generated by custom-written software and PCAMP10 (Molecular Devices Corp). Briefly the protocol is constituted of three parts: a test pulse P1 at –20 mV to assess IiNa, steps of 10 mV from –60 mV to +50 mV increase to allow Ca2+ to enter the cell and a final test pulse P2 at –20 mV to evaluate the effect of Ca2+ on IiNa. The length of P1 and P2 were 15 ms, the steps for Ca2+ entry were 200 ms in length. The holding potential was –120 mV. Capacitance compensation was optimized and series resistance was compensated by 40–80% Membrane currents were filtered at 5 KHz and digitized with 12-bit resolution through a DigiData-1200 interface (Molecular Devices Corp.).
Expression and purification of Na<sub>v</sub>1.4 CTerm-CaM complex. The gene for <i>H. sapiens</i> Na<sub>v</sub>1.4 CTerm Long (SCNA4; aa 1599–1764; ENF…GYD) in a pGEX-6-P1 plasmid with a N-terminal GST tag was purchased from Genscript, together with the gene for full-length mammalian calmodulin (<i>R. norvegicus</i> CALM2; 100% amino acid identity with <i>H. sapiens</i> CaM) cloned into a pET24b plasmid<sup>3</sup> were used to co-transform BL21-CodonPlus RIL E. coli (Agilent) cells. The proteins were co-expressed and purified as described. The supernatant was loaded onto a 30 ml Glutathione Sepharose 4 Fast Flow resin using gravity flow. The column was washed with 200 ml buffer and protein was eluted in aliquots of 8 ml with an elution buffer containing 154 mg of reduced L-glutathione in 50 mM Tris, 1 mM EDTA, 2 mM DTT, pH 8.0. Eluted fractions containing protein were pooled and 5 µg of PreScission protease was added per mg of NaV CTerm and CaM complex. This construct contained an additional 7 residues at the C-terminus, LTRAAAS. The supernatant was loaded onto a HiPrep 16/10 column (GE Lifesciences) equilibrated in a matched buffer using 10 mM CaCl<sub>2</sub>, 2 mM DTT, pH 7.4. The buffer was changed 3 times and the final dialysis was allowed to proceed overnight. The dialyzed and Precision-cleaved protein was loaded on a Source Q anion exchange column. Elution was performed using a buffer of 20 mM Tris and 1 mM DTT and a gradient of 50–500 mM NaCl. Free cleaved tag eluted at ~8 mM NaCl. CaM and CaM in complex eluted at ~100 mM NaCl. Fractions were judged to be 98% pure by SDS-PAGE gel then pooled and concentrated to ~40 mg/ml protein. This gene for <i>Homo sapiens</i> Na<sub>v</sub>1.4 CTerm Short (SCNA4; aa 1599–1754; ENF…YMY) was cloned into a pGEX-6-P1 plasmid with an N-terminal GST tag. The supernatant was loaded onto a 30 ml Glutathione Sepharose 4 Fast Flow resin using gravity flow. The column was washed with 200 ml buffer and protein was eluted in aliquots of 8 ml with an elution buffer containing 154 mg of reduced L-glutathione in 50 mM Tris, 1 mM EDTA, 2 mM DTT, pH 7.4. The buffer was changed 3 times and the final dialysis was allowed to proceed overnight. This construct contained an additional 7 residues at the C-terminus, LTRAAAS. BL21-CodonPlus RIL E. coli (Agilent) were then co-transformed with Na<sub>v</sub>1.4 CTerm Short and CALM2 PET-24b plasmids<sup>4</sup>. The proteins were co-expressed and purified in complex in the same manner as the Na<sub>v</sub>1.4 CTerm Long and CaM complex.

Synthesis of DIII linker peptides. The 12-mer peptide corresponding to Na<sub>v</sub>1.4 residues 1316–1327 (100% identity with Na<sub>v</sub>1.5 residues 1491–1502) was synthesized with an addition of 2 N-terminal alanines (AAAKQKYNAMKGLL) at the N-terminus. This peptide has 25% identity with the helical portion of the DIII–DIV linker seen contacting the EFL in NavPa<sub>6</sub> (PDB ID: 5x 0 M<sup>2</sup>). Synthesis was performed by AnaSpec and the peptide was judged to be over 90% pure by HPLC. The 20-mer peptide containing Na<sub>v</sub>1.4 residues 1512–1533 (100% identity with Na<sub>v</sub>1.5 residues 1487–1506) was synthesized (MTEEQKQYNNAMKGLS SKPQP). This peptide has 30% identity with the DIII–DIV linker seen in NavPa<sub>6</sub>. Synthesis was performed by GenScript and judged to be over 95% pure.

Structures of Na<sub>v</sub>1.4 CTerm-CaM complex<sup>11</sup>. The Na<sub>v</sub>1.4 CTerm Long in complex with apo CaM (50 µM EGTA) was crystallized by hanging drop vapor diffusion at 18°C with a 1:1 ratio of protein to well solution<sup>29</sup>. The structure was determined by molecular replacement with Phaser<sup>35</sup> using as a search model the Na<sub>v</sub>1.4 CTerm–CaM complexes (PDB ID: 4OVN, chain I and D, CaM residues 82–147) followed by the independent placement of the N-lobe (PDB 1K93, chain D, CaM residues 57–66)<sup>36</sup>. One copy of the CaM–CTerm complex was placed in the asymmetric unit. Model building was completed with iterative cycles of manual rebuilding with Coot<sup>30</sup> and refinement with Phenix<sup>36</sup>. The structure was re-fitted to an R<sub>free</sub> of 23.2 (R = 27.9) with excellent geometry (Table 1). Buried surface areas (BSA) were calculated with PISA<sup>14</sup>. <i>The Na<sub>v</sub>1.4 CTerm Short (residues 1599–1754) in complex with CaM was crystallized by hanging drop vapor diffusion at 18°C with a 1:1 ratio of protein to well solution<sup>29</sup>. The structure was determined by molecular replacement with Phaser<sup>35</sup> using as a search model the Na<sub>v</sub>1.4 CTerm complex (PDB ID: 4OVN) as CaM lobe was the most successful. The CTerm of the Na<sub>v</sub>1.4 CTerm–CaM complex was placed in the asymmetric unit. Model building was completed with iterative cycles of manual rebuilding with Coot<sup>30</sup> and refinement with Phenix<sup>36</sup>. The final structure was re-fitted to an R<sub>free</sub> of 23.8 (R = 29.7) with excellent geometry (Table 1).

 Isothermal calorimetry titration experiments. Titration experiments were conducted using a VP-ITC MicroCalorimeter (MicroCal Inc.) and data were analyzed with the Origin-5.0 software and fitted to a single binding site per monomer and the error of this fit is reported. The concentration of CTerm used was always between 2 and 25 nM. The concentration of CaM was varied from 17.5 µM to 1.5 µM depending on the calculated dissociation constant. The results of one replicate are reported in Table 2 and Supplementary Table 1. Proteins were prepared for ITC titrations by 3 rounds of dialysis; two of 4-h and one overnight. The buffer used was 20 mM MOPS, 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 50 µM EGTA with 1 mM CaCl<sub>2</sub> added for high calcium titrations. A thermodynamic model was built consisting of two sequential reaction schemes. The first reaction (Fig. 4a) is the thermodynamic cycle of CaM binding Ca<sup>2+</sup>. The binding of two Ca<sup>2+</sup> ions to either CaM lobe was modeled as a single event and single-ion-bound intermediates were not considered. This model contains four states: apoCaM, (Ca<sup>2+</sup>)<sub>2</sub>–CaM, (Ca<sup>2+</sup>)<sub>2</sub>–CaM<sup>2+</sup> and CaM<sup>2+</sup>–CaM. The E<sub>Ca</sub> values for the two Ca<sup>2+</sup> binding steps<sup>18</sup> to either lobe in full-length mammalian CaM was reported by Evans and Shea as −12.82 ± 0.99 kcal mol<sup>−1</sup> for the N-lobe and −15.06 ± 0.03 kcal mol<sup>−1</sup> for the C-lobe and Linse et al. as −12.7 ± 0.6 and −14.9 ± 0.2 kcal mol<sup>−1</sup>. Comparing the thermodynamic model results generated with the two sets of binding constants revealed some differences of CaM data from Evans and Shea were used on the first binding cycle (Fig. 4a) of the thermodynamic model to match the inclusion of 1 mM MgCl<sub>2</sub> in our experimental buffer.

Ca<sup>2+</sup>–uncaging electrophysiology experiments. Whole-cell patch clamp experiments, HEK293 (ATCC CRL-1573) cells were cultured on 10-cm plates, and channels transiently transfected by calcium phosphate method<sup>40</sup>. To construct NaV1.5 AP-QJ channel, we PCR amplified the NaV1.5 CT using the forward (5′-ATATACTTGGTGTACAGGGTCGTTTTTTGCCGTACAGGGTCGTGTC-3′) and reverse (5′-TCTTGGTGCACGAGTTTGACAGGGTCGTTTTTTGCCGTACAGGGTCGTGTC-3′) primers (Supplementary Table 3). Subsequently, the amplified DNA segment was digested using KpnI and XbaI restriction enzyme sites and ligated into the N-terminus of the DIII–DIV linker seen contacting the EFL in NavPa<sub>6</sub> (PDB ID: 5x 0 M<sup>2</sup>). Synthesis was performed by AnaSpec and the peptide was judged to be over 90% pure by HPLC. The 20-mer peptide containing NaV1.5 residues 1491–1506 was synthesized with an addition of 2 N-terminal alanines (AAAKQKYNAMKGLL) at the N-terminus. This peptide has 30% identity with the DIII–DIV linker seen in NavPa<sub>6</sub>. Synthesis was performed by GenScript and judged to be over 95% pure.

Structures of Na<sub>v</sub>1.4 CTerm-CaM complexes. The Na<sub>v</sub>1.4 CTerm Long in complex with apo CaM (50 µM EGTA) was crystallized by hanging drop vapor diffusion at 18°C with a 1:1 ratio of protein to well solution<sup>29</sup>. The structure was determined by molecular replacement with Phaser<sup>35</sup> using as a search model the Na<sub>v</sub>1.4 CTerm–CaM complexes (PDB ID: 4OVN, chain I and D, CaM residues 82–147) followed by the independent placement of the N-lobe (PDB 1K93, chain D, CaM residues 57–66)<sup>36</sup>. One copy of the CaM–CTerm complex was placed in the asymmetric unit. Model building was completed with iterative cycles of manual rebuilding with Coot<sup>30</sup> and refinement with Phenix<sup>36</sup>. The structure was re-fitted to an R<sub>free</sub> of 23.2 (R = 27.9) with excellent geometry (Table 1). Buried surface areas (BSA) were calculated with PISA<sup>14</sup>.
full-length Nav1.5. For Ca2+-uncaging experiments, we applied 8 µg of CDNA encoding either R. norvegicus wild type or NaV1.5 AP-IQ channel (residues 1–1922) in 1 µl of CaCl2-containing media. The above CDNA constructs were included within mammalian expression plasmids driven by a cytomegalovirus promoter. To boost expression, CDNA for simian virus 40T antigen (1–2 µg) was co-transfected. Cultures were probed −1–2 days following transfection.

For Ca2+-uncaging experiments, internal solution contained (in mM): TEA-MeSO3, 45; HEPES (pH 7.4), 10; NaCl, 100; at 300 mOsm, adjusted with 558 KCl with a 545/40BP filter for detecting Fluo4FF, and a 580LP filter for detecting Ca2+. Typically, for flashes in range 0.5–2 µm, DMN, 1 mM; and CaCl2, 0.7 mM. For the 2–8 µm range, DMN, 2 mM; and CaCl2, 1.4 mM. For larger Ca2+ steps, DMN, 4 mM; and CaCl2, 3 mM. Since DMN can bind Mg2+, all experiments were conducted with 0 mM Mg2+ internally. The bath solution (in mM) contained: TEA-MeSO3, 45; HEPES (pH 7.4), 10; NaCl, 100; at 300 mOsm, adjusted with TEA-MeSO3.

All Ca2+-uncaging experiments were conducted on a Nikon TE2000 inverted microscope with a Plan Fluor Apo 40 × oil objective. Ca2+ was delivered by a Cre recombinase-based UV-photolysis system. UV pulses of ~1.5 ms were delivered from an Xe arc lamp. Typically, for experiments using DEVD or Fluo4FF, and a 580LP filter for detecting Ca2+, the Nature Research Reporting Summary linked to this article. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article. To boost expression, cDNA for simian virus 40T antigen (1 µg) was co-transfected. Cultures were probed −1–2 days following transfection.

For Ca2+-uncaging experiments, internal solution contained (in mM): TEA-MeSO3, 45; HEPES (pH 7.4), 10; NaCl, 100; at 300 mOsm, adjusted with TEA-MeSO3. The source data underlying Fig. 5a, b, 5e and 5g was deposited in the Protein Data Bank. The Nature Research Reporting Summary linked to this article. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article. For Ca2+-uncaging experiments, internal solution contained (in mM): TEA-MeSO3, 45; HEPES (pH 7.4), 10; NaCl, 100; at 300 mOsm, adjusted with TEA-MeSO3.
Acknowledgements
This work was funded by NIH NHLBI (HL128743). X-ray data collection was carried out at beamlines FMX and AMX, part of the Life Science Biomedical Technology Research resource (LSBR) primarily supported by the National Institute of Health, National Institute of General Medical Sciences (NIGMS) through a Biomedical Technology Research Resource P41 grant (P41GM111244), and by the DOE Office of Biological and Environmental Research (KP165010). As a National Synchrotron Light Source II facility resource at Brookhaven National Laboratory, work performed at the LSBR is supported in part by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences Program under contract number and DE-SC0012704 (KCO401040). Special thanks to Richard W. Aldrich (UT Austin) for helpful discussion and insightful comments and suggestions. We thank Rahul Banerjee, Sara Nathan, and Mofeed Nagib for helpful discussions.

Author contributions
Binding experiments and structural determination was performed by J.B.Y. with help from L.M.A., L.S., and S.R.S.; NaV chimera electrophysiology experiments were performed by F.F.; NaV truncation electrophysiology experiments were performed by M.B.J. Thermodynamic analysis was performed by J.B.Y. and L.M.A.; structural model building was performed by J.B.Y., S.B.G., and L.M.A.; electrophysiology analysis was performed by M.B.J., F.F., and G.L.T. The manuscript was written by J.B.Y. and L.M.A. with editing by all authors.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-09570-7.

Competing interests: The authors declare no competing interests.

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Journal peer review information: Nature Communications thanks Zhiguang Yuchi, Christopher Ahern and the other anonymous reviewer(s) for their contribution to the peer review of this work.

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