



Hierarchical CRMP2 posttranslational modifications control NaV1.7 function

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Edited by William A. Catterall, University of Washington School of Medicine, Seattle, WA, and approved November 7, 2016 (received for review June 28, 2016)

Voltage-gated sodium channels are crucial determinants of neuronal excitability and signaling. Trafficking of the voltage-gated sodium channel NaV1.7 is dysregulated in neuropathic pain. We identify a trafficking program for NaV1.7 driven by hierarchical interactions with posttranslationally modified versions of the binding partner collapsin response mediator protein 2 (CRMP2). The binding described between CRMP2 and NaV1.7 was enhanced by conjugation of CRMP2 with small ubiquitin-like modifier (SUMO) and further controlled by the phosphorylation status of CRMP2. We determined that CRMP2 SUMOylation is enhanced by prior phosphorylation by cyclin-dependent kinase 5 and antagonized by Fyn phosphorylation. As a consequence of CRMP2 loss of SUMOylation and binding to NaV1.7, the channel displays decreased membrane localization and current density, and reduces neuronal excitability. Preventing CRMP2 SUMOylation with a SUMO-impaired CRMP2-K374A mutant triggered NaV1.7 internalization in a clathrin-dependent manner involving the E3 ubiquitin ligase Nedd4-2 (neural precursor cell expressed developmentally down-regulated protein 4) and endocytosis adaptor proteins Numb and epidermal growth factor receptor pathway substrate 15. Collectively, our work shows that diverse modifications of CRMP2 cross-talk to control NaV1.7 activity and illustrate a general principle for regulation of NaV1.7.

NaV1.7 sodium channel | trafficking | CRMP2 | SUMOylation | phosphorylation

The tetrodotoxin-sensitive (TTX-S) voltage-gated sodium channel NaV1.7 generates thresholds for action potential firing in sensory neurons. Genetic and functional studies have established NaV1.7 as a major contributor to pain signaling in humans and have demonstrated that mutations in *SCN9A*, the gene encoding NaV1.7, produce distinct human pain syndromes (1–4). Pain also results from up-regulated NaV1.7 expression (5–7); however, dysregulation of NaV1.7 is poorly understood.

In search of NaV1.7 trafficking/regulatory events, we reported that surface expression and current density of NaV1.7 was controlled by SUMOylation of the cytosolic axonal collapsin response mediator protein 2 (CRMP2) (8). CRMP2 regulates multiple processes in neurons and was initially discovered to regulate mechanisms of neuronal polarity (9, 10). CRMP2 phosphorylation by cyclin-dependent kinase 5 (Cdk5) (11), glycogen synthase kinase 3 β (10), Rho-associated protein kinase (12), or the Src-family kinases Fyn (13) and Yes (14) drives its diverse cellular functions, including neurite outgrowth, endocytosis, and ion-channel trafficking (8, 15–17). Studies of CRMP2 trafficking functions have revealed that CRMP2 facilitates endocytosis of L1-cell adhesion molecule by interacting with the endocytic protein Numb (18) that recruits epidermal growth factor receptor pathway substrate 15 (Eps15), an initiator of clathrin-mediated endocytosis (19).

In neuropathic pain, NaV1.7 surface localization is augmented by loss of Nedd4-2 (neural precursor cell expressed developmentally down-regulated protein 4), an E3 ubiquitin ligase that labels NaV1.7 for endocytosis (6). Inspired by these observations, we tested the hypothesis that a cross-talk between distinct CRMP2 posttranslational modifications is a key factor in determining

NaV1.7 trafficking and localization. Here, we (i) demonstrate a pathway of NaV1.7 regulation dependent upon CRMP2 SUMOylation and phosphorylation states, (ii) identify interactions between CRMP2 and NaV1.7, and (iii) map molecular determinants of NaV1.7 endocytosis that result from altered CRMP2 interactions. We also report that the CRMP2-NaV1.7 signaling is conserved between rodent and human sensory neurons, supporting the exciting possibility that this pathway could be the target of future therapeutic agents aimed at controlling NaV1.7 in patients with neuropathic pain.

Results

CRMP2 Is Necessary for Maintaining TTX-S Sodium Currents in Sensory Neurons. If CRMP2 loss of SUMOylation inhibits NaV1.7 activity, a similar reduction in NaV1.7 currents should be seen if CRMP2 expression is reduced, particularly if CRMP2 is necessary for maintaining NaV1.7 currents. NaV1.7 currents can be analyzed by whole-cell patch-clamp electrophysiology of mouse catecholamine A differentiated (CAD) cells, a central nervous system-derived catecholaminergic cell line that expresses high levels of NaV1.7 (20) and isolated dorsal root ganglia (DRG) sensory neurons responsible for transmission of noxious stimuli from glabrous skin through the sciatic nerve and to the spinal cord. A decrease in CRMP2 expression (Fig. S1A) caused a reduction in TTX-S sodium currents in rat DRG neurons (Fig. 1A, B) and in NaV1.7 currents in CAD cells without altering biophysical properties of the channels (Fig. S1 B–E and Table S1). Notably,

Significance

The voltage-gated sodium channel NaV1.7 is important for electogenesis in sensory neurons. Insertion within the membrane is required for function of NaV1.7. However, the mechanisms determining how NaV1.7 is trafficked to neuronal cell membranes are poorly understood. Here, we elucidate a signaling program involving a complex and intriguing posttranslational modification regime of collapsin response mediator protein 2 (CRMP2), an NaV1.7-binding protein. NaV1.7 surface localization and currents are controlled by CRMP2 modifications. Activity of NaV1.7 is thought to modulate neuronal excitability that codes for several sensory modalities, including chronic pain, as inferred from human pain disorders caused by mutations in NaV1.7 channels. Understanding the role of cross-talk between CRMP2 modifications in modulation of NaV1.7 activity opens routes to exploit this system for pain.

Author contributions: E.T.D., A.M., M.K., and R.K. designed research; E.T.D., A.M., X.Y., and Y.W. performed research; A.M. and R.K. contributed new reagents/analytic tools; E.T.D., A.M., X.Y., Y.W., and R.K. analyzed data; and E.T.D., A.M., M.K., and R.K. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1610531113/-DCSupplemental.

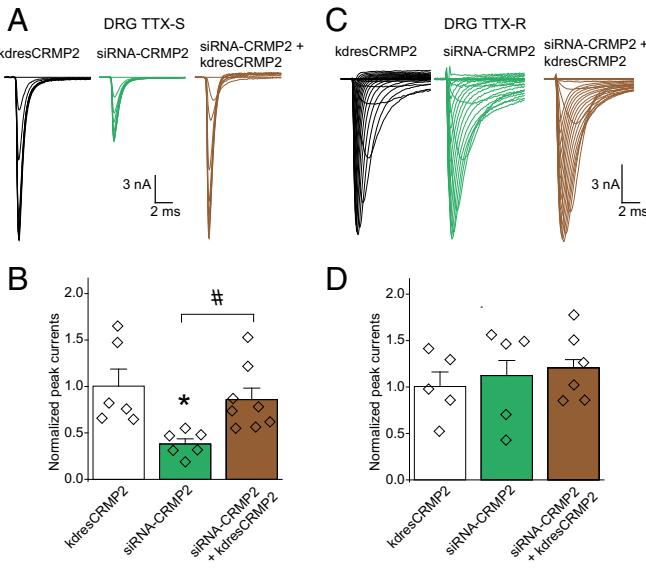


Fig. 1. CRMP2 is necessary for maintaining TTX-S sodium currents in sensory neurons. Sodium current traces representing TTX-S (*A*) and TTX-R (*C*) currents. Quantitative analysis of TTX-S (*B*) and TTX-R (*D*) DRG peak sodium current densities (picoamps/picofarad; pA/pF). DRG currents were 13.8 ± 4.2 nA and recorded from cells with conductances of 28.9 ± 9.1 pF. Unless otherwise indicated, for this and subsequent figures, all data represent mean \pm SEM. Individual data points and *n* values are indicated by diamonds overlaid on the bar graphs [*n* = 5–7; **P* < 0.05 vs. knockdown-resistant (kdres) CRMP2; #*P* < 0.05, Kruskal-Wallis test with Dunnett's post hoc comparisons]. Voltage protocols and post hoc subtraction used to investigate DRG cell peak TTX-R and TTX-S currents are described in *S1 Materials and Methods*.

sodium currents were normalized by “add-back” of a CRMP2 refractory to knockdown (Figs. 1*A* and *B* and *S1 A–C*), confirming that CRMP2 is necessary for selectively maintaining TTX-S sodium currents.

Interplay Between CRMP2 Modifications Selectively Alter NaV1.7 Trafficking. A loss of CRMP2 expression via knockdown (Fig. 1) or loss of CRMP2 SUMOylation via expression of SUMO-impaired mutant reduces sodium currents (8), but whether CRMP2 phosphorylation events also modulate sodium currents is unknown. To examine possible priming by CRMP2 modifications in modulation of NaV1.7 and to identify the relationships between CRMP2 modifications and their effects on NaV1.7, CRMP2 mutants with SUMO-site impaired, phospho-null sites, or both (Fig. 2*A*) were engineered. Consistent with previous findings (8), loss of CRMP2 SUMOylation reduced NaV1.7 surface fraction and currents (Fig. 2*B*, *C*, and *E*). NaV1.7 surface localization and current density were reduced by mutations in CRMP2 at the site phosphorylated by Cdk5 (i.e., S522A) but not sites phosphorylated by other kinases [Fyn (Y32F), Yes (Y479F), GSK-3β (T509A/A514A), RhoK (T555A)]; Fig. 2*B*, *C*, and *E*). Expression of WT CRMP2 or SUMO-impaired (i.e., K374A), or phospho-deficient mutants did not alter NaV1.7 protein expression (Fig. 2*D*). Concomitant elimination of CRMP2 SUMOylation and phosphorylation (Cdk5 site) did not further decrease NaV1.7 surface fraction or current compared with that observed with either mutation alone (Fig. 2*B*, *C*, and *E*), suggesting a convergent mechanism of modulation. Concomitant elimination of CRMP2 SUMOylation and Fyn phosphorylation had no effect on NaV1.7 surface fraction or currents (Fig. 2*B*, *C*, and *E*), suggesting that Fyn phosphorylation of CRMP2 is obligatory for eliciting the negative regulation induced by loss of CRMP2 SUMOylation. These results illustrate that at least three different posttranslational modifications of

CRMP2, namely SUMOylation, Cdk5 phosphorylation, and Fyn phosphorylation, collaborate to control NaV1.7 trafficking and currents.

The reduction in DRG sodium currents imposed by loss of CRMP2 SUMOylation and loss of Cdk5 phosphorylation was limited to NaV1.7, as no changes were observed in TTX-resistant (TTX-R) currents (predominantly NaV1.8; Fig. 3*A*) or in non-NaV1.7 TTX-S (predominantly NaV1.1 and NaV1.6) currents upon their isolation with electrophysiological and pharmacological protocols (Fig. 3*A*). Additionally, hNaV1.1, rNaV1.3, or the cardiac hNaV1.5 expressed in heterologous cells were also unaffected by loss of CRMP2 SUMOylation (8) or phosphorylation (Table S1).

CRMP2 Modifications Control Excitability of DRG Neurons. NaV1.7 contributes to DRG excitability as evident in human gain-of-function mutations that increase action potential firing (21), a phenomenon directly correlated to and underlying transmission of painful stimuli. Consequently, we tested excitability properties of DRGs expressing SUMO-impaired or Cdk5 phospho-null CRMP2. Loss of these CRMP2 modifications reduced evoked action potential frequency by half (Fig. 3*B*). Rheobase, the current required to initiate an action potential, was increased in cells expressing the Cdk5 phospho-null CRMP2; however, interpretation of this result as dependent on CRMP2 SUMOylation status or NaV1.7 trafficking is made difficult by previously described relationships between CRMP2 phosphorylation by Cdk5 and modulation of N-type voltage-gated calcium (CaV2.2) channels that may also modulate rheobase (22) (Fig. 3*C*). The resting membrane potential, action potential spike height, and action potential half width were equivalent among all conditions (Fig. 3 *D–F*). These findings strengthen the hypothesis that CRMP2 modifications are linked to NaV1.7 activity.

Biochemical Mapping of CRMP2 Modifications and Binding to NaV1.7. Next, we asked if CRMP2 modifications influence each other to choreograph CRMP2’s activity toward NaV1.7. Eliminating CRMP2 SUMOylation did not affect CRMP2 phosphorylation (Fig. 4 *A* and *B*). In contrast, elimination of the CRMP2 Cdk5 phosphorylation site prevented CRMP2 SUMOylation in CAD cells (Fig. 4 *C* and *D*). Additionally, Cdk5, together with its co-factor p25, facilitated in vitro SUMOylation of recombinant CRMP2 (Fig. 4*E*). Finally, loss of CRMP2 SUMOylation alone or together with loss of Cdk5 phosphorylation of CRMP2 reduced NaV1.7–CRMP2 binding (Fig. 4 *F* and *G*). Together, these observations place Cdk5-mediated phosphorylation of CRMP2 upstream of SUMOylation to control NaV1.7 surface expression, current density, and DRG neuron excitability.

Interfering with SUMOylation and Cdk5-Mediated Phosphorylation of CRMP2 Promotes Clathrin-Mediated Endocytosis of NaV1.7. CRMP2 SUMOylation controls surface, but not total, NaV1.7 expression (Fig. 2 *B–D*), suggesting that CRMP2 can relocalize the channel away from the membrane to produce reduced current densities. Because CRMP2 interacts with the endocytic adaptor Numb (18) to regulate L1-cell adhesion molecule internalization via clathrin-mediated endocytosis (19), we asked if loss of CRMP2 SUMOylation prevents NaV1.7 membrane localization by inducing its endocytosis. Pitstop2, a clathrin assembly inhibitor (23), prevented current density reductions imposed by loss of CRMP2 SUMOylation or Cdk5 phosphorylation (Fig. 5*A*), arguing for an involvement of clathrin-mediated endocytosis in NaV1.7 regulation by CRMP2. The internalized NaV1.7 relocalized to early (Fig. 5 *B* and *C*) and recycling (Fig. S2 *A* and *B*) endosomal compartments when these CRMP2 modifications were lost. The proteasome inhibitor Lactacystin did not rescue the reduction in NaV1.7 current imposed by loss of CRMP2 modifications (Fig. S2*C*), thus ruling out a degradation of the channel. These results identify a trafficking route

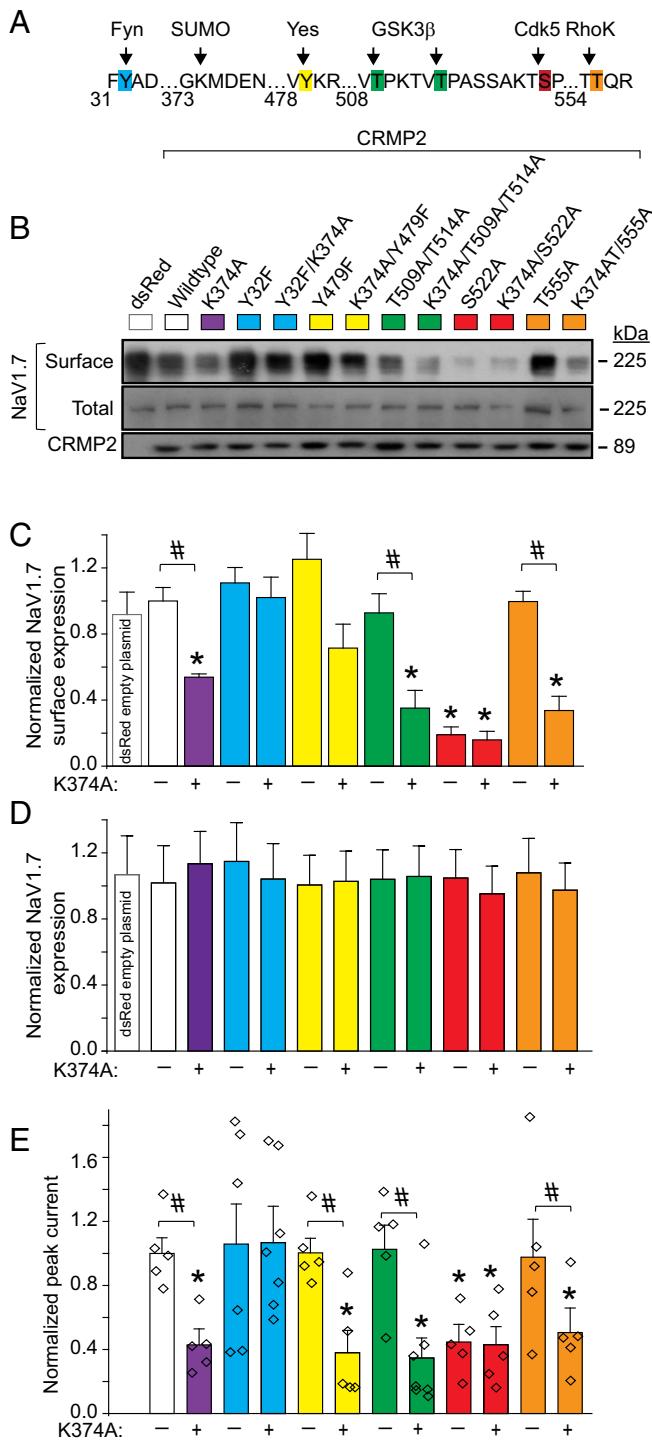


Fig. 2. Expression of SUMOylation- and phosphorylation-deficient CRMP2 plasmids regulates NaV1.7 surface expression and current density in mouse CAD cells. (A) Amino acid sequence of CRMP2 highlighting sites of phosphorylation and SUMOylation. Numbers refer to amino acids of rat CRMP2. (B) Representative immunoblots of streptavidin-enriched surface fraction probed with a NaV1.7 antibody (*Top*) and total fraction probed with a pan-NaV antibody (*Middle*). (*Bottom*) Blot of lysates probed with CRMP2 antibody to verify expression of exogenous ~89-kDa CRMP2 ($n = 5$). (C) Bar graphs summarizing mean surface NaV1.7 in CAD cells transfected with the indicated plasmids. Data are normalized to total NaV1.7 protein and then to the CRMP2 in each lane and plotted as ratio of the WT CRMP2 condition. (D) Bar graphs summarizing mean total NaV1.7 in CAD cells transfected with the indicated plasmids. Data are normalized to total NaV1.7 protein and then to β -tubulin in each lane and plotted as ratio of the WT CRMP2 condition.

followed by NaV1.7 under control of CRMP2 modifications and demonstrate that CRMP2's activity toward NaV1.7 involves clathrin-mediated endocytosis.

CRMP2 Recruits an Endocytic Complex to Down-Regulate NaV1.7.

Having demonstrated that CRMP2 regulates endocytosis of NaV1.7, we next investigated the molecular mechanism involved. CRMP2 reportedly controls trafficking of membrane proteins and vesicles (8, 15, 18). As NaV1.7 endocytosis requires mono-ubiquitination by the ubiquitin ligase Nedd4-2 (24) and Numb—a CRMP2 protein partner acts as a scaffold between Nedd4 (25) and the endocytic machinery (19)—we hypothesized that recruitment of these proteins by CRMP2 may promote NaV1.7 internalization. Additionally, Numb can recruit Eps15 (19, 26, 27). Together, these proteins compose the machinery required to mark a protein for endocytosis (28) and initiate clathrin-mediated endocytosis by enabling interactions between Eps15 and a mono-ubiquitinated cargo. To test this hypothesis, we examined CRMP2 interactions with endocytic proteins Numb and Eps15 and Numb-interacting E3 ubiquitin ligases Nedd4-2 and Itch (29). Loss of CRMP2 SUMOylation, but not Cdk5-mediated phosphorylation or simultaneous loss of both modifications, increased CRMP2's association with each of these proteins (Fig. 5 *D* and *E*). If these CRMP2-interacting proteins participate in NaV1.7 internalization, eliminating their expression should prevent CRMP2-mediated reductions of current density. Indeed, following specific reduction of these CRMP2-interacting proteins (Fig. S3 *A* and *B*), the inhibition in NaV1.7 currents forced by loss of CRMP2 modifications was rescued (Fig. 5*F*). Taken together, our data support the conclusion that CRMP2 association with proteins Numb, Eps15, and Nedd4-2 triggers endocytosis of NaV1.7 (Fig. 5*G*).

Hierarchical Interactions Between CRMP2 Modifications Modulate NaV1.7 Endocytosis.

To further investigate the underlying mechanism by which CRMP2 Cdk5 phosphorylation and SUMOylation control NaV1.7 endocytosis and current density, Cdk5 phosphorylation levels were enhanced with a Cdk5 site phospho-mimetic CRMP2 (i.e., pseudophosphorylated) mutant or overexpression of Cdk5 itself. CRMP2 SUMOylation was unaffected by mimicking constitutive phosphorylation by Cdk5 (Figs. 6 *A* and *B* and S4 *A* and *B*). Whereas CRMP2 loss of SUMOylation decreased NaV1.7-CRMP2 interaction (Fig. 4 *F* and *G*), mimicking constitutive phosphorylation by Cdk5 prevented this effect (Figs. 6 *A* and *B* and S4 *A* and *B*). Supporting these results, mimicking constitutive Cdk5 phosphorylation in CAD cells and in DRG neurons prevented loss of NaV1.7 currents imposed by non-SUMOylated CRMP2 (Figs. 6 *C* and *D* and S4 *C*). Finally, inhibition of NaV1.7 currents by a Cdk5 phosphorylation-deficient CRMP2 mutant (CRMP2-S522A; Figs. 2*E* and 3*A*) was refractory to rescue by simultaneous Cdk5 overexpression (Fig. S4 *C*). These outcomes demonstrate dominance of CRMP2 Cdk5 phosphorylation over SUMOylation in control of NaV1.7 currents.

Cdk5 phosphorylation of CRMP2 could regulate NaV1.7 trafficking by preventing assembly of a CRMP2-endocytic complex. To test this hypothesis, interactions between CRMP2 and Numb, Eps15, or Nedd4-2 were examined. Mimicking constitutive phosphorylation of CRMP2 by Cdk5 (Fig. 6 *E* and *F*) and overexpression of Cdk5 inhibited the CRMP2–Numb interaction (Fig. S4 *D* and *E*). Furthermore, enhanced interactions between non-SUMOylated CRMP2 and Numb, Eps15, or Nedd4-2 (Fig. 5 *D* and *E*) were prevented by constitutive phosphorylation of

dition. (E) Quantitative analysis of CAD cell peak current density following expression of indicated CRMP2 plasmids ($n = 5$ –7). CAD cell currents throughout this study averaged 1.3 ± 0.1 nA and were recorded from cells with conductances of 21.2 ± 1.7 pF.

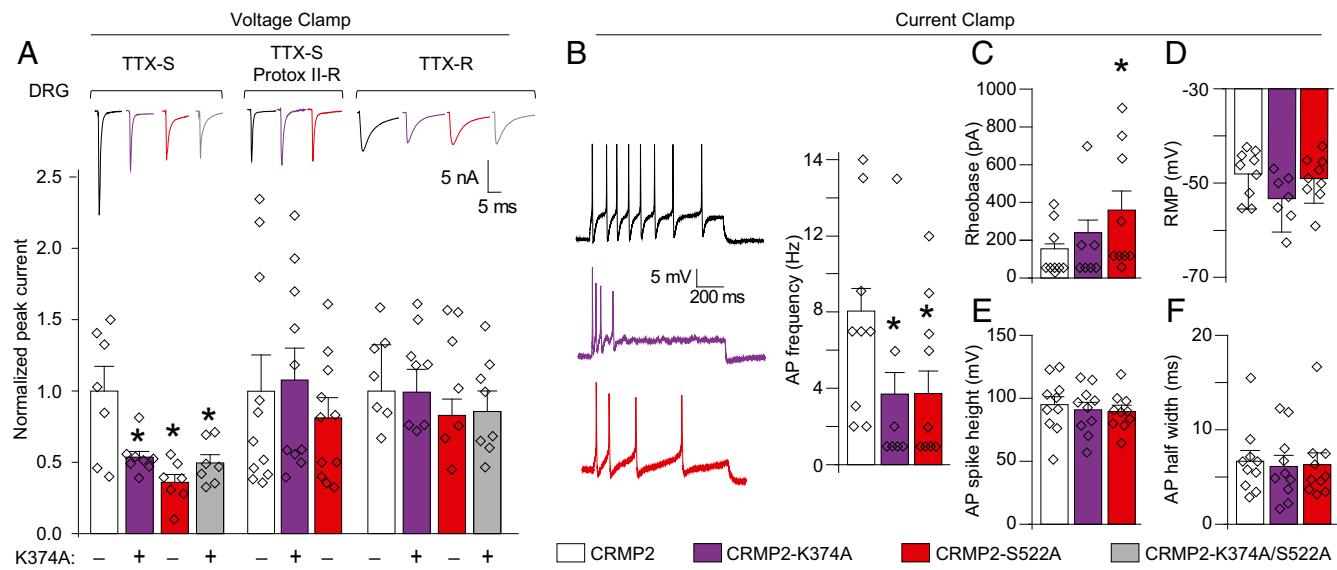


Fig. 3. CRMP2 modifications control NaV1.7 currents and excitability (*A*). Normalized peak sodium current density (picoamps/picofarad; pA/pF) from DRG neurons transfected with WT (CRMP2), SUMO-impaired (CRMP2-K374A), phospho-null (CRMP2-S522A), or dual SUMO-impaired/phospho-null (CRMP2-K374A/S522A) CRMP2 ($n = 7$ –11). Representative traces are displayed above the bar graphs. Total sodium current was separated electrically and pharmacologically into TTX-S (NaV1.1, NaV1.6, NaV1.7), TTX-S/Protox-II-resistant (NaV1.1, NaV1.6; 5 nM Protox-II), and TTX-R (NaV1.8; 500 nM TTX). DRG currents were 13.9 ± 2.7 nA and recorded from cells with conductances of 33.3 ± 6.6 pF ($n = 7$ –11). Summary of current-clamp properties: action potentials (AP) and representative traces (*B*), rheobase (*C*), resting membrane potential (RMP) (*D*), action potential spike height (*E*), and action potential half width (*F*) ($n = 7$ –10 per condition; * $P < 0.05$ vs. WT CRMP2; Kruskal-Wallis test with Dunnett's post hoc comparisons).

CRMP2 by Cdk5 (Fig. 6 *E* and *F* and Fig. S4 *D* and *E*). These findings identify that a non-SUMOylated, nonphosphorylated (by Cdk5) CRMP2 recruits Numb to trigger NaV1.7 endocytosis (Fig. 6*G*) and illustrate that CRMP2's activity on NaV1.7 is negatively regulated by Cdk5 phosphorylation of CRMP2.

Antagonism Between CRMP2 Fyn Phosphorylation and SUMOylation Contributes to NaV1.7 Endocytosis. Simultaneous loss of Fyn phosphorylation and SUMOylation of CRMP2 overcame the NaV1.7 current reduction imposed by loss of SUMOylation alone (Figs. 2*E*

and 7*A*). Although loss of Fyn phosphorylation did not change CRMP2's SUMOylation state (Fig. S5*A*), it prevented the loss of binding to NaV1.7 imposed by loss of SUMOylation (Fig. S5*B*). Concomitant loss of Fyn phosphorylation and SUMOylation of CRMP2 suppressed the internalization of NaV1.7 imposed by loss of SUMOylation alone (Fig. 2 *B* and *C*). Loss of Fyn phosphorylation prevented the increased binding to Numb, Eps15, and Nedd4-2 imposed by loss of CRMP2 SUMOylation (Fig. S5 *C* and *D*). Thus, loss of Fyn phosphorylation is dominant over CRMP2 SUMOylation in the control of NaV1.7 internalization.

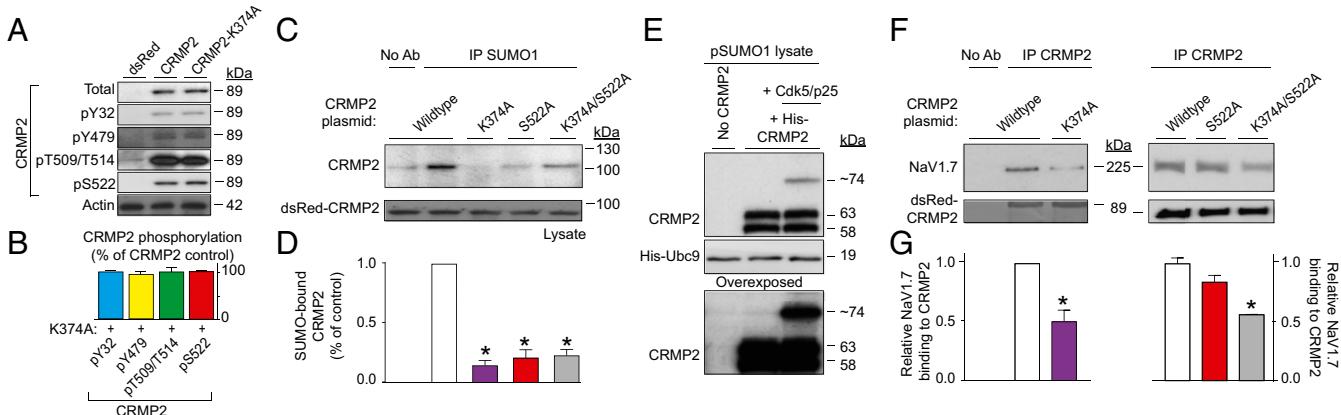


Fig. 4. CRMP2 SUMOylation status is affected by its phosphorylation, and both modifications contribute to NaV1.7 binding. (*A*) Representative Western blot of CRMP2 and CRMP2-K374A expressing CAD cells indicating CRMP2 phosphorylation levels at indicated kinase target sites ($n = 3$). No signal was detected with a CRMP2 antibody against pThreonine-555 ($n = 3$). (*B*) Quantitative analysis of CRMP2-K374A phosphorylation compared with total CRMP2. Representative immunoblot (*C*) and summary (*D*) to detect SUMOylated CRMP2 from CAD cells transfected with indicated plasmids ($n = 3$). (*E*) Representative immunoblot of in vitro SUMOylation of CRMP2. A SUMOylated CRMP2 band (~74 kDa) was detected only when Cdk5 and its cofactor p25 were added to the reaction. The E2 SUMO-conjugating enzyme Ubc9, the key component of protein SUMOylation, is unchanged by the kinase. The overexposed blot shows no SUMOylated CRMP2 when the Cdk5/p25 complex is not present. Representative immunoblot (*F*) and summary (*G*) of immunoprecipitation (IP) with CRMP2 to detect NaV1.7 from CAD cells transfected with indicated plasmids ($n = 6$). Asterisks indicate statistically significant differences compared with WT CRMP2-expressing control cells ($P < 0.05$, one-way ANOVA with Tukey's post hoc test; $n = 3$).

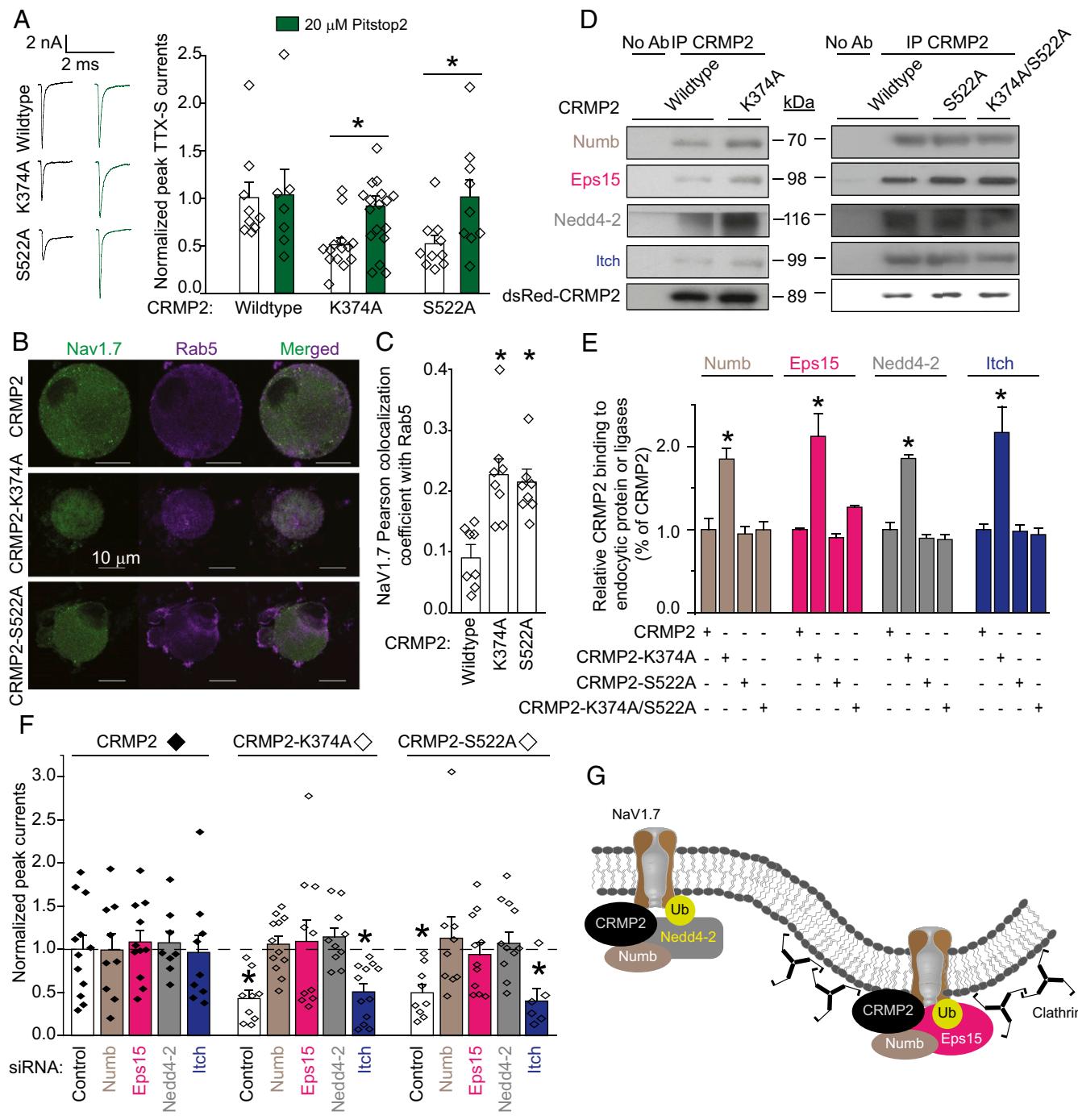


Fig. 5. CRMP2 modifications control its interactions with endocytosis-related proteins, NaV1.7 trafficking, and endocytosis via clathrin-mediated endocytosis. (A) Normalized peak TTX-S sodium current density (picoamps/picofarad; pA/pF) from DRG neurons transfected with CRMP2, CRMP2-K374A, or CRMP2-S522A before and 30 min after application of 20 μM of Pitstop2, a clathrin-mediated endocytosis inhibitor. Representative traces are displayed adjacent to the bar graphs ($n = 7-17$). (B) Representative confocal images of DRG neurons costained for NaV1.7 and the endosome marker Rab5. (Scale bar, 10 μm.) (C) Summary of Pearson correlation coefficient of NaV1.7 and Rab5 containing ($n = 8$). (D) Representative immunoblots (D) and summary (E) of CRMP2 IP from CAD cells transfected with the indicated plasmids and probed with antibodies for endocytosis proteins Numb and Eps15 and E3 ubiquitin ligases Nedd4-2 and Itch ($n = 3$). (F) Normalized peak TTX-S sodium current density (pA/pF) from DRG neurons transfected with indicated plasmids and siRNAs to knock down Numb, Eps15, Nedd4-2, or Itch. DRG currents were 11.3 ± 0.8 nA and recorded from cells 36.0 ± 2.6 pF ($n = 6-12$; * $P < 0.05$ vs. CRMP2 control, Kruskal-Wallis test with Dunnnett's post hoc comparisons). (G) Schematic of proposed mechanism of CRMP2-mediated endocytosis of NaV1.7. Non-SUMOylated CRMP2 binds to the endocytic protein Numb to recruit the E3 ligase Nedd4-2, which monoubiquitinates NaV1.7 (Left). Recruitment of Eps15 to this complex elicits clathrin-mediated endocytosis of NaV1.7 (Right).

To further resolve the hierarchy of CRMP2 posttranslational modifications in control of NaV1.7, we asked if loss of Fyn phosphorylation could be dominant over loss of Cdk5 phosphorylation of CRMP2. NaV1.7 current reductions induced by (i) SUMO-impaired

CRMP2 mutant; (ii) SUMO proteases SENP1/2, which remove SUMO from conjugated proteins; or (iii) loss of Cdk5-mediated phosphorylation were prevented when Fyn phosphorylation was also eliminated (Fig. S5E). Additionally, a dominant-negative Fyn

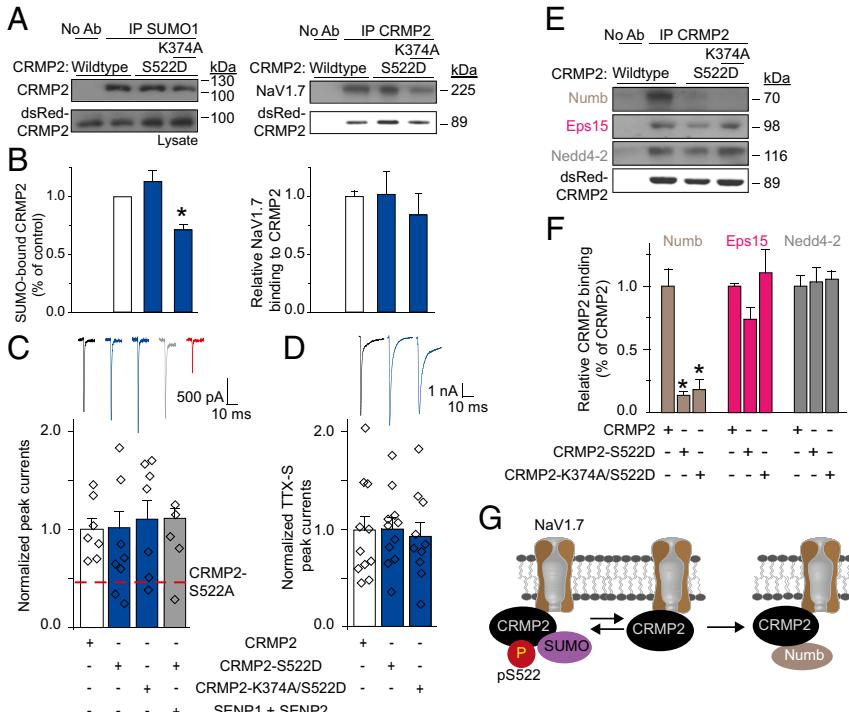


Fig. 6. Constitutive Cdk5 phosphorylation of CRMP2 overrides CRMP2 deSUMOylation-mediated loss of NaV1.7 current density via changes in interactions with endocytic machinery. Representative immunoblots (A) and summary (B) of IP with SUMO1 or CRMP2 antibodies from CAD cells transfected with the indicated plasmids and probed with antibodies against CRMP2 (Left) or NaV1.7 (Right; $n = 3$). (C) Representative traces and normalized peak sodium current density (picoamps/picofarad; pA/pF) from CAD cells ($n = 5-8$) or TTX-S current density from DRGs ($n = 10-11$) (D) transfected with the indicated plasmids. DRG currents were 9.9 ± 1.8 nA in amplitude and recorded from cells with average conductances of 30.0 ± 5.3 pF. For comparison, the red dashed line, representing data from Fig. 2E, is illustrated in C. Representative immunoblots (E) and summary (F) of IP with CRMP2 antibody from CAD cells transfected with the indicated plasmids and probed with antibodies against Numb, Eps15, Nedd4-2, and CRMP2 ($n = 3$; * $P < 0.05$ vs. CRMP2 WT, Kruskal-Wallis test with Dunnett's post hoc comparisons). (G) Model proposing that elimination of Cdk5-mediated CRMP2 phosphorylation promotes CRMP2's interaction with Numb.

kinase (DNFyn-K299M) (30) normalized the current density reductions observed with loss of CRMP2 SUMOylation or phosphorylation (Cdk5 site; Figs. 7B and S5F). Therefore, loss of Fyn phosphorylation of CRMP2 dominates all CRMP2-dependent posttranslational modifications that reduce NaV1.7 currents.

To determine the mechanism by which Fyn phosphorylation of CRMP2 regulates NaV1.7, we next investigated CRMP2 SUMOylation and CRMP2's interactions with NaV1.7 and endocytosis proteins. Overexpression of a competent Fyn kinase increased CRMP2 phosphorylation (at Y32), decreased CRMP2 SUMOylation (Fig. 7C), decreased CRMP2's interaction with NaV1.7 (Fig. 7D), and increased CRMP2's interaction with the endocytic scaffolding protein Eps15 (Fig. 7E and F). Because loss of CRMP2 SUMOylation translates into a reduction in NaV1.7 current density (Fig. 2E), likely as a result of a decrease in binding to NaV1.7 (Fig. 4F and G), we hypothesized that Fyn-phosphorylated CRMP2 would lead to a suppression of NaV1.7 currents in CAD cells and in DRG neurons. As expected, forcing Fyn phosphorylation of CRMP2 led to a specific reduction in NaV1.7 currents compared with cells expressing WT CRMP2 (Fig. 7G and H). Forcing Fyn phosphorylation of a SUMO-incompetent CRMP2 did not result in any additional reduction in NaV1.7 currents (Fig. 7G). Conversely, coexpression of competent Fyn kinase and a Fyn phosphorylation-incompetent CRMP2 rescued baseline NaV1.7 current densities (Fig. 7G), directly implicating Fyn phosphorylation of CRMP2 in control of NaV1.7 activity.

Next, we manipulated Cdk5 phosphorylation of CRMP2 to further dissect the interplay between CRMP2 posttranslational modifications in control of NaV1.7. Loss of Cdk5 phosphorylation of CRMP2 together with forcing Fyn phosphorylation did

not result in a further decrease of NaV1.7 currents, even when the SUMOylation site was deleted (Fig. 7G). On the contrary, gain of Cdk5 phosphorylation of CRMP2 was dominant over the gain of Fyn phosphorylation of CRMP2 (Fig. 7G), and this effect occurred regardless of CRMP2's SUMOylation status. Loss of NaV1.7 currents imposed by forcing Fyn phosphorylation of WT CRMP2 in DRG neurons was normalized by inhibition of clathrin mediated endocytosis with Pitstop2 (Fig. 7H). Under no conditions were the biophysical properties of NaV1.7 channels changed (Table S1). Thus, CRMP2-dependent internalization of NaV1.7 requires the simultaneous gain of Fyn phosphorylation coupled with loss of Cdk5 phosphorylation of CRMP2, which result in reduced SUMOylation of CRMP2 (Fig. 7I) and NaV1.7 endocytosis (Fig. 7J). These three posttranslational modifications coordinate CRMP2's activity toward NaV1.7.

CRMP2 Modulation of NaV1.7 Currents Is Maintained in Human DRGs. The relationship between CRMP2 posttranslational modifications and NaV1.7 currents in human DRGs has not been defined. Validating the relationships between CRMP2 posttranslational modifications and NaV1.7 trafficking in humans is critical to understanding the mechanistic underpinnings of channel dysregulation in human disease and the eventual translation of cell signaling studies to pre-clinical models. Therefore, human DRGs were obtained and transfected with SUMO-impaired or phospho-null CRMP2 mutants (Fig. 8A), and sodium currents were recorded. Appropriately compensated recordings did not reveal any differences in biophysical channel properties (Fig. S6 A-C), and use-dependence was unaffected (Fig. S6 D and E). Importantly, both the total (Fig. 8 B and C) and the TTX-S fraction (Fig. 8 D and E) of sodium currents were

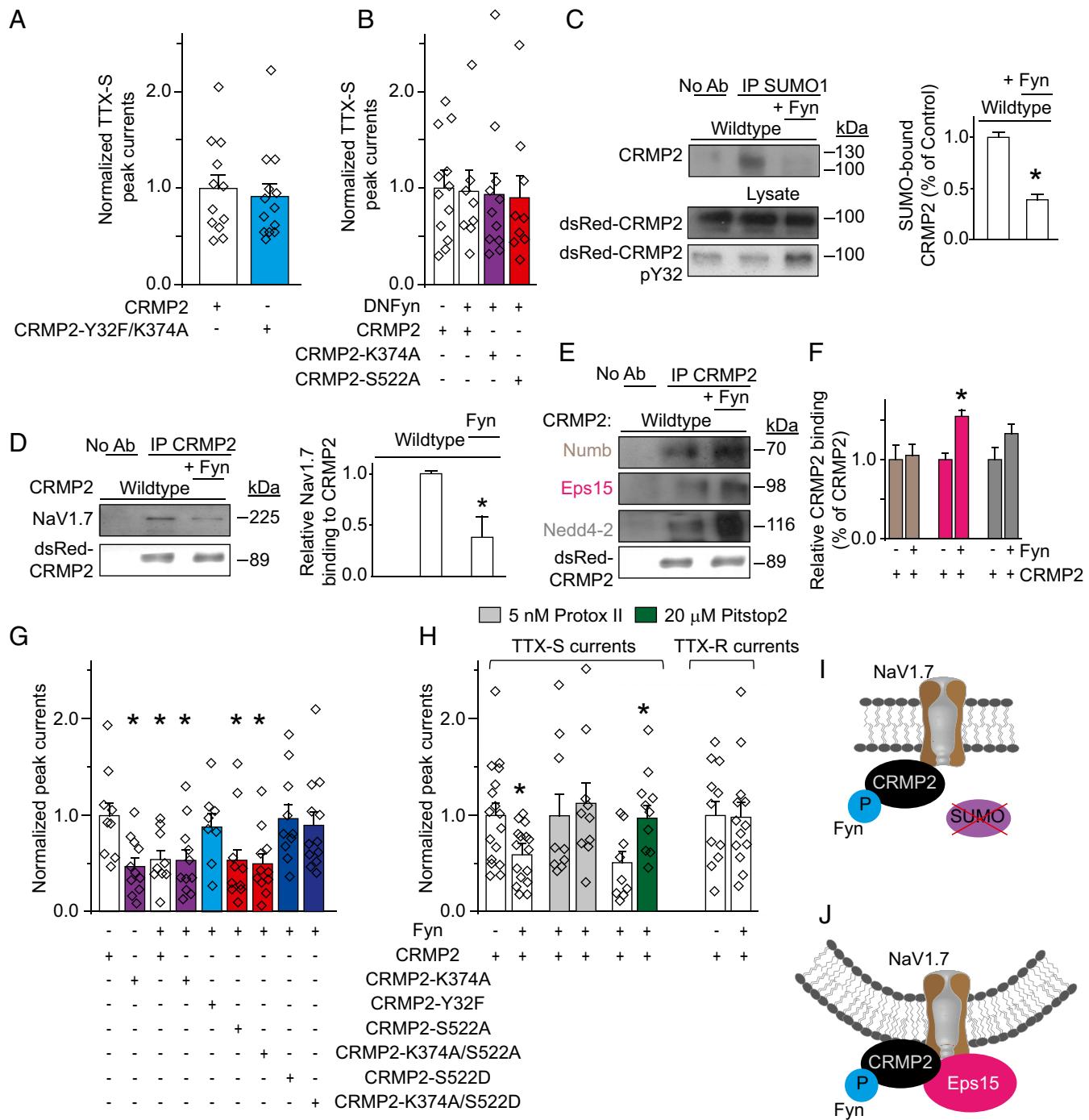


Fig. 7. Gain of CRMP2 phosphorylation by Fyn overrides the reduction in Nav1.7 current density imposed by loss of CRMP2 SUMOylation or CRMP2 phosphorylation by Cdk5. Normalized peak TTX-S current density (picoamps/picofarad; pA/pF) (A and B) from DRGs transfected with WT or the double Fyn-null/SUMO-impaired (CRMP2-Y32F/K374A) CRMP2 ($n = 12-13$) (A) or the inactive dominant-negative Fyn kinase ($n = 8-12$) in addition to the indicated CRMP2 plasmids (B). (C) Representative immunoblots (Left) and summary (Right) of denaturing IP with SUMO1 antibody from CAD cells transfected with the indicated plasmids and probed with CRMP2 antibody. Lysates were also probed for expression of dsRed-CRMP2 (Middle) and pY32 CRMP2 (Bottom; $n = 3$). (D) Representative immunoblots (Left) and summary (Right) of IP with CRMP2 antibody from CAD cells transfected with the indicated plasmids and probed with a NaV1.7 antibody ($n = 3$). Representative immunoblots (E) and summary (F) of IP with CRMP2 antibody from CAD cells transfected with the indicated plasmids and probed with antibodies for Numb, Eps15, Nedd4-2, and CRMP2 ($n = 3$). Normalized peak sodium current density (pA/pF) from transfected CAD cells ($n = 8-12$) (G) or TTX-S, non-NaV1.7-TTX-S fraction, TTX-S fraction following Pitstop2 application (20 μ M, 30 min), or TTX-R sodium current density from DRGs ($n = 9-17$) (H). DRG currents were 10.3 ± 1.5 nA in amplitude and recorded from cells with average conductances of 34.6 ± 5.6 pF (* $P < 0.05$ vs. CRMP2 WT, Kruskal-Wallis test with Dunnett's post hoc comparisons). (I) Model proposing that Fyn-mediated CRMP2 phosphorylation impairs CRMP2 SUMOylation, but enhances its interaction with Eps15 (J).

decreased by loss of these CRMP2 modifications in human DRGs. The congruence between CRMP2 modulation of sodium currents in rat and human DRGs validates the CRMP2-NaV1.7 signaling model

(Fig. 9 A and B) and provides a strong mechanistic understanding from which future studies can target CRMP2-NaV1.7 interactions in preclinical animal models of human pain syndromes.

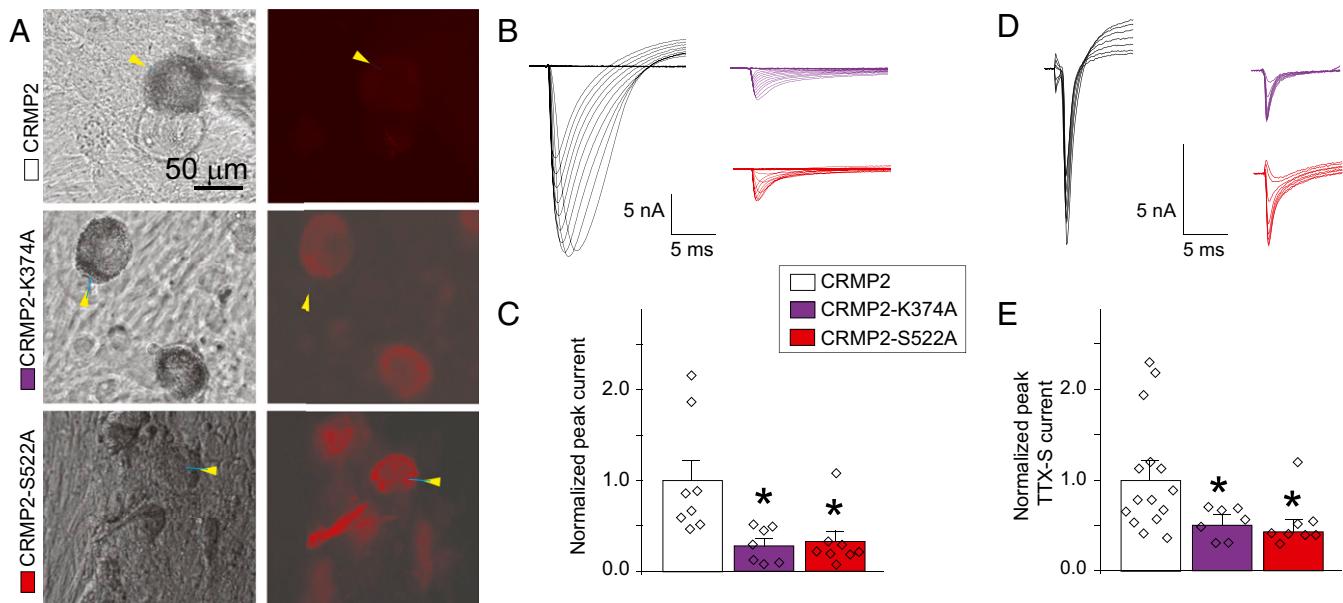


Fig. 8. CRMP2 modifications control NaV1.7 currents in human DRGs. (A) Representative differential interference contrast (Left) and fluorescence (dsRed; Right) images showing transfection of human DRGs (yellow arrowheads). Representative families of total sodium current traces (B) or TTX-S sodium current traces (D) from human DRGs expressing WT, SUMO-impaired (CRMP2-K374A), or phospho-null (CRMP2-S522A) CRMP2. Normalized peak total sodium current density ($n = 7-8$) (C) or TTX-S sodium current density [picoamps/picofarad (pA/pF); $n = 7-15$] (E) from human DRGs expressing the indicated plasmids. Human DRG currents were 23.1 ± 4.0 nA and recorded from cells with average conductances of 84.3 ± 16.5 pF (* $P < 0.05$ vs. CRMP2 WT, Kruskal-Wallis test with Dunnett's post hoc comparisons).

Discussion

The present findings make a compelling case for targeting CRMP2 posttranslational modifications, loss of which selectively attenuates NaV1.7 currents and dampens nociceptor excitability, as a pathway for development of therapeutic agents for the treatment of neuropathic pain. Although CRMP2 posttranslational modifications serve to direct protein activity to a variety of functional cellular processes, CRMP2 SUMOylation does not feed back onto CRMP2 phosphorylation as evident by unchanged CRMP2 phosphorylation between WT and K374A mutant CRMP2 (Fig. 4A and B). Therefore, although posttranslational modifications codify

a molecular language that instruct CRMP2 to positively or negatively influence NaV1.7 surface expression, a direct intervention of CRMP2 SUMOylation has potential to attenuate pain states without modifying CRMP2 phosphorylation or other functions of CRMP2. It may also be possible that the CRMP2-NaV1.7 trafficking pathway dissected in our work (Fig. 9), which is congruent between rodent and human DRGs, is dysfunctional within human disease pain states and contributes to nociceptor excitability. Future studies will aim to dissect if subtle tuning of the CRMP2 modifications that alter NaV1.7 currents can result in significant reduction of pain in animal models.

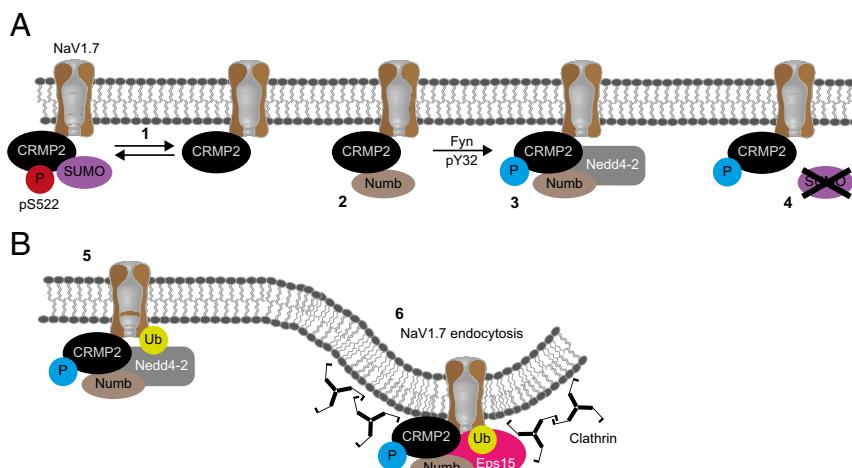


Fig. 9. Schematic of CRMP2 modifications and their contribution to the regulation of NaV1.7 trafficking. (A) CRMP2 SUMOylation (1) is prevented when CRMP2 phosphorylation by Cdk5 at S522 is also prevented. Consequently, nonSUMOylated CRMP2 has an enhanced interaction with Numb (2). The enhanced interaction with Numb and subsequent phosphorylation of CRMP2 by Fyn kinase (3) can facilitate further recruitment of the E3 ubiquitin ligase Nedd4-2. Furthermore, when phosphorylated by Fyn, CRMP2 SUMOylation is prevented (4). (B) Within the CRMP2/NaV1.7/endocytic machinery complex, Nedd4-2 monoubiquitinates NaV1.7 (5), which, in turn, permits Eps15/clathrin-mediated endocytosis of the channel (6).

From a mechanistic perspective, the NaV1.7 trafficking pathway identified here greatly extends the findings of Laedermann et al., who reported that the Nedd4-2 ubiquitin ligase monoubiquitinates NaV1.7 to signal the channel's endocytosis (6). Our results suggest that induction of membrane curvature by Eps15 (26, 27) to trigger clathrin-mediated endocytosis is required for CRMP2-mediated endocytosis of NaV1.7 via the formation of a CRMP2/Numb/Eps15/Nedd4-2 complex. If CRMP2 is not SUMOylated, these interactions are strengthened, culminating in accumulation of the channel into early and recycling endosomes. This study also extends our own previous work that demonstrated a link between NaV1.7 function and CRMP2 SUMOylation (8) to now illustrate that the trafficking program of NaV1.7 is dependent on multiple modifications of CRMP2 and details the mechanism of endocytosis of NaV1.7.

Akin to the relationship described here between CRMP2 and NaV1.7, several studies have been successful in identifying links between protein SUMOylation and trafficking of an accessory protein. For instance, the immediate early gene Activity-regulated cytoskeleton-associated protein/Activity-regulated gene 3.1 (Arc), which couples changes in neuronal activity to synaptic plasticity events (31), is SUMOylated (32). Arc SUMOylation impacts the trafficking of AMPARs, which are heterotetrameric glutamate-gated ion channels that underpin the vast majority of fast excitatory glutamate neurotransmission in the central nervous system (33). Preventing Arc SUMOylation may control AMPARs. Rab3-Interacting Molecule 1 α (RIM1 α) is a presynaptic protein implicated in the docking/priming of synaptic vesicles and in the development of short- and long-term synaptic plasticity (34, 35). RIM1 α SUMOylation is required for presynaptic targeting and clustering of Cav2.1 calcium channels (36). Our study adds to the growing evidence that protein SUMOylation is critical for trafficking processes in neurons.

A salient finding of our work is the selectivity in CRMP2 modulation of NaV1.7, but not other channels. How CRMP2 regulates NaV1.7 specifically is a critical question. One possibility may be differential recruitment of the endocytic machinery or CRMP2 itself. Nedd4 proteins regulate trafficking of multiple sodium channels (6, 37) whereas Numb and Eps15 proteins are general players in clathrin-mediated endocytosis (18, 19, 27), so specificity is unlikely endowed by these proteins. Likely, the specificity is conferred by CRMP2, as its expression and modifications causally influence NaV1.7. CRMP2 may have differential binding affinities for NaV1.x channels with the highest affinity for NaV1.7. Although this binding has not been mapped for each of the channels, a hypothesis to explain selective regulation of NaV1.7 may be differential binding of CRMP2 to intracellular loops of sodium channels, especially those that are not conserved between NaV1.x isoforms (e.g., the N terminus and loop 2 connecting the second and third transmembrane domain modules). Because CRMPs are heterotetrameric, we cannot rule out contributions to channel specificity by other CRMP isoforms. For instance, CRMP4 expression increases following sciatic nerve axotomy (38), and a CRMP2–CRMP4 tetramer may permit binding to NaV1.7, but not other NaV1.x channels. CRMP4-KO mice exhibit impaired olfactory ability (39), and loss-of-function mutations in NaV1.7 cause anosmia (40), thus providing further evidence of a possible link between CRMP4 and NaV1.7. However, another possibility is that there may be a still unknown protein that is required for the CRMP2-NaV1.7 specificity.

Many proteins bear multiple, distinct modifications, and the ability of one modification to antagonize or synergize the addition

of another can have significant biological consequences. Of the multiple kinases that target CRMP2, it is interesting that phosphorylation by Cdk5 and Fyn confers regulatory checkpoints upon CRMP2 SUMOylation that steer CRMP2/NaV1.7 signaling in opposing directions (Fig. 9). Notably, both kinases have been linked to pain. Increased Cdk5 activity has been reported in inflammatory (41) and neuropathic (42) pain models, and roscovitine, a Cdk5-inhibiting compound, blunted neuropathic pain after chronic compression injury of DRGs (42). Constitutive action of Fyn produced mechanical allodynia and thermal hyperalgesia via enhanced expression of ionotropic glutamate receptors (43). Despite the breadth of pain research and continuous identification of novel targets of protein SUMOylation, no previous reports have linked this modification to pain as far as we are aware. A weak link can be surmised from data demonstrating an enhanced susceptibility to symptomatic knee osteoarthritis and multiple regional pain in patients who show expression of the NaV1.7 R1150W mutation (44) and an increase in global SUMOylation rate in rheumatoid arthritis (45).

NaV1.7 is a high-value target for development of new pain therapeutic agents. Tarantula peptide toxins are selective for NaV1.7 in vitro but fail to provide analgesia in pain models (46), likely because of a lack of penetration past the perineurial barrier (47). A NaV1.7-selective centipede-venom peptide elicited anti-nociception in acute pain models but has not yet been tested in more complex rodent pain models (48). A monoclonal antibody against the voltage sensor of NaV channels exhibited selectivity for NaV1.7, with partial effects on NaV1.6 (49), and several small-molecule blockers of human NaV1.7 have been described (50). Thus, strategies targeting NaV1.7 channels are ongoing and show promise in pain therapy. Disrupting CRMP2 SUMOylation is an approach to controlling NaV1.7 channels that overcomes the selectivity hurdle of targeting NaV1.7 directly. When posttranslationally modified, CRMP2 reduces NaV1.7 currents and excitability. Thus, modification of CRMP2 by SUMOylation is a mechanism to selectively regulate NaV1.7, and interacting with this pathway to curb NaV1.7 activity could normalize nociceptor excitability. From a translational perspective, the concordance in NaV1.7 regulation by CRMP2 in rodent and human sensory neurons will likely mean better success when this strategy is pursued in future clinical trials.

Materials and Methods

Detailed descriptions of methods used and any associated references are available in *SI Materials and Methods*. Briefly, all electrophysiology and biochemistry experiments were performed according to established protocols (8, 15). All animal protocols were approved by the institutional animal care and use committee of the College of Medicine at the University of Arizona and conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health.

ACKNOWLEDGMENTS. We thank Dr. Yoshio Goshima (Yokohama City University Graduate School of Medicine, Yokohama, Japan) for providing CRMP2-pY32 antibody and Dr. Pascale Giraudon (Lyon Neuroscience Research Center, Lyon, France) for providing CRMP2-pY479 antibody; members of the R. Khanna laboratory for technical assistance regarding this project, and Isabel N. Angeles for assistance with plasmid cloning. This work was supported by a Career Development Award from the Arizona Health Science Center (to M.K.), National Scientist Development Grant SDG5280023 from the American Heart Association (to R.K.), Neurofibromatosis New Investigator Award NF1000099 from the Department of Defense Congressionally Directed Military Medical Research and Development Program (to R.K.), and a Young Investigator Award from the Children's Tumor Foundation (to A.M.).

1. Dib-Hajj SD, Yang Y, Waxman SG (2008) Genetics and molecular pathophysiology of Na(v)1.7-related pain syndromes. *Adv Genet* 63:85–110.
2. Jarecki BW, Sheets PL, Jackson JO, 2nd, Cummins TR (2008) Paroxysmal extreme pain disorder mutations within the D3/S4-S5 linker of Nav1.7 cause moderate destabilization of fast inactivation. *J Physiol* 586(17):4137–4153.
3. Waxman SG (2007) Nav1.7, its mutations, and the syndromes that they cause. *Neurology* 69(6):505–507.
4. Bennett DL, Woods CG (2014) Painful and painless channelopathies. *Lancet Neurol* 13(6):587–599.
5. Hong S, Morrow TJ, Paulson PE, Isom LL, Wiley JW (2004) Early painful diabetic neuropathy is associated with differential changes in tetrodotoxin-sensitive and -resistant sodium channels in dorsal root ganglion neurons in the rat. *J Biol Chem* 279(28):29341–29350.
6. Laedermann CJ, et al. (2013) Dysregulation of voltage-gated sodium channels by ubiquitin ligase NEDD4-2 in neuropathic pain. *J Clin Invest* 123(7):3002–3013.

7. Zhang H, Dougherty PM (2014) Enhanced excitability of primary sensory neurons and altered gene expression of neuronal ion channels in dorsal root ganglion in paclitaxel-induced peripheral neuropathy. *Anesthesiology* 120(6):1463–1475.
8. Dustrude ET, Wilson SM, Ju W, Xiao Y, Khanna R (2013) CRMP2 protein SUMOylation modulates NaV1.7 channel trafficking. *J Biol Chem* 288(34):24316–24331.
9. Fukata Y, et al. (2002) CRMP-2 binds to tubulin heterodimers to promote microtubule assembly. *Nat Cell Biol* 4(8):583–591.
10. Yoshimura T, et al. (2005) GSK-3beta regulates phosphorylation of CRMP-2 and neuronal polarity. *Cell* 120(1):137–149.
11. Cole AR, et al. (2006) Distinct priming kinases contribute to differential regulation of collapsin response mediator proteins by glycogen synthase kinase-3 in vivo. *J Biol Chem* 281(24):16591–16598.
12. Arimura N, et al. (2000) Phosphorylation of collapsin response mediator protein-2 by Rho-kinase. Evidence for two separate signaling pathways for growth cone collapse. *J Biol Chem* 275(31):23973–23980.
13. Uchida Y, et al. (2009) Semaphorin3A signaling mediated by Fyn-dependent tyrosine phosphorylation of collapsin response mediator protein 2 at tyrosine 32. *J Biol Chem* 284(40):27393–27401.
14. Varrin-Doyer M, et al. (2009) Phosphorylation of collapsin response mediator protein 2 on Tyr-479 regulates CXCL12-induced T lymphocyte migration. *J Biol Chem* 284(19):13265–13276.
15. Brittain JM, et al. (2011) Suppression of inflammatory and neuropathic pain by uncoupling CRMP-2 from the presynaptic Ca²⁺ channel complex. *Nat Med* 17(7):822–829.
16. Moutal A, François-Moutal L, Brittain JM, Khanna M, Khanna R (2015) Differential neuroprotective potential of CRMP2 peptide aptamers conjugated to cationic, hydrophobic, and amphiphatic cell penetrating peptides. *Front Cell Neurosci* 8:471.
17. Brustovetsky T, Pellman JJ, Yang XF, Khanna R, Brustovetsky N (2014) Collapsin response mediator protein 2 (CRMP2) interacts with N-methyl-D-aspartate (NMDA) receptor and Na⁺/Ca²⁺ exchanger and regulates their functional activity. *J Biol Chem* 289(11):7470–7482.
18. Nishimura T, et al. (2003) CRMP-2 regulates polarized Numb-mediated endocytosis for axon growth. *Nat Cell Biol* 5(9):819–826.
19. Santolini E, et al. (2000) Numb is an endocytic protein. *J Cell Biol* 151(6):1345–1352.
20. Wang Y, et al. (2011) Development and characterization of novel derivatives of the antiepileptic drug lacosamide that exhibit far greater enhancement in slow inactivation of voltage-gated sodium channels. *ACS Chem Neurosci* 2(2):90–106.
21. Dib-Hajj SD, Yang Y, Black JA, Waxman SG (2013) The Na(V)1.7 sodium channel: from molecule to man. *Nat Rev Neurosci* 14(1):49–62.
22. Brittain JM, Wang Y, Eruvvetere O, Khanna R (2012) Cdk5-mediated phosphorylation of CRMP-2 enhances its interaction with CaV2.2. *FEBS Lett* 586(21):3813–3818.
23. von Kleist L, et al. (2011) Role of the clathrin terminal domain in regulating coated pit dynamics revealed by small molecule inhibition. *Cell* 146(3):471–484.
24. Laedermann CJ, Decosterd I, Abriel H (2014) Ubiquitylation of voltage-gated sodium channels. *Handb Exp Pharmacol* 221:231–250.
25. Kawahashi K, Sakata T, Hayashi S (2007) Modulation of Notch signal transduction by endocytic regulators Numb and the Nedd4 family of ubiquitin ligases. *A Dros Res Conf* 48:444C.
26. Woelk T, et al. (2006) Molecular mechanisms of coupled monoubiquitination. *Nat Cell Biol* 8(11):1246–1254.
27. Horvath CA, Vanden Broeck D, Boulet GA, Bogers J, De Wolf MJ (2007) Epsin: inducing membrane curvature. *Int J Biochem Cell Biol* 39(10):1765–1770.
28. Polo S, et al. (2002) A single motif responsible for ubiquitin recognition and mono-ubiquitination in endocytic proteins. *Nature* 416(6879):451–455.
29. Di Marcotullio L, et al. (2006) Numb is a suppressor of Hedgehog signalling and targets Gli1 for Itch-dependent ubiquitination. *Nat Cell Biol* 8(12):1415–1423.
30. Osterhout DJ, Wolven A, Wolf RM, Resh MD, Chao MV (1999) Morphological differentiation of oligodendrocytes requires activation of Fyn tyrosine kinase. *J Cell Biol* 145(6):1209–1218.
31. Bramham CR, et al. (2010) The Arc of synaptic memory. *Exp Brain Res* 200(2):125–140.
32. Craig TJ, et al. (2012) Homeostatic synaptic scaling is regulated by protein SUMOylation. *J Biol Chem* 287(27):22781–22788.
33. Chowdhury S, et al. (2006) Arc/Arg3.1 interacts with the endocytic machinery to regulate AMPA receptor trafficking. *Neuron* 52(3):445–459.
34. Dulubova I, et al. (2005) A Munc13/RIM/Rab3 tripartite complex: from priming to plasticity? *EMBO J* 24(16):2839–2850.
35. Castillo PE, Schoch S, Schmitz F, Südhof TC, Malenka RC (2002) RIM1alpha is required for presynaptic long-term potentiation. *Nature* 415(6869):327–330.
36. Girach F, Craig TJ, Rocca DL, Henley JM (2013) RIM1α SUMOylation is required for fast synaptic vesicle exocytosis. *Cell Reports* 5(5):1294–1301.
37. Fotia AB, et al. (2004) Regulation of neuronal voltage-gated sodium channels by the ubiquitin-protein ligases Nedd4 and Nedd4-2. *J Biol Chem* 279(28):28930–28935.
38. Jang SY, et al. (2010) Injury-induced CRMP4 expression in adult sensory neurons: A possible target gene for ciliary neurotrophic factor. *Neurosci Lett* 485(1):37–42.
39. Tsutaya A, Nishihara M, Goshima Y, Ohtani-Kaneko R (2015) Mouse pups lacking collapsin response mediator protein 4 manifest impaired olfactory function and hyperactivity in the olfactory bulb. *Eur J Neurosci* 42(6):2335–2345.
40. Weiss J, et al. (2011) Loss-of-function mutations in sodium channel Nav1.7 cause anosmia. *Nature* 472(7342):186–190.
41. Yang YR, et al. (2007) Activation of cyclin-dependent kinase 5 (Cdk5) in primary sensory and dorsal horn neurons by peripheral inflammation contributes to heat hyperalgesia. *Pain* 127(1–2):109–120.
42. Yang L, Gu X, Zhang W, Zhang J, Ma Z (2014) Cdk5 inhibitor roscovitine alleviates neuropathic pain in the dorsal root ganglia by downregulating N-methyl-D-aspartate receptor subunit 2A. *Neurol Sci* 35(9):1365–1371.
43. Liu YN, Yang X, Suo ZW, Xu YM, Hu XD (2014) Fyn kinase-regulated NMDA receptor- and AMPA receptor-dependent pain sensitization in spinal dorsal horn of mice. *Eur J Pain* 18(8):1120–1128.
44. Valdes AM, et al. (2011) Role of the Nav1.7 R1150W amino acid change in susceptibility to symptomatic knee osteoarthritis and multiple regional pain. *Arthritis Care Res (Hoboken)* 63(3):440–444.
45. Meinecke I, et al. (2007) Modification of nuclear PML protein by SUMO-1 regulates Fas-induced apoptosis in rheumatoid arthritis synovial fibroblasts. *Proc Natl Acad Sci USA* 104(12):5073–5078.
46. Theile JW, Cummins TR (2011) Recent developments regarding voltage-gated sodium channel blockers for the treatment of inherited and acquired neuropathic pain syndromes. *Front Pharmacol* 2:54.
47. Schmalhofer WA, et al. (2008) ProTx-II, a selective inhibitor of NaV1.7 sodium channels, blocks action potential propagation in nociceptors. *Mol Pharmacol* 74(5):1476–1484.
48. Yang S, et al. (2013) Discovery of a selective NaV1.7 inhibitor from centipede venom with analgesic efficacy exceeding morphine in rodent pain models. *Proc Natl Acad Sci USA* 110(43):17534–17539.
49. Lee JH, et al. (2014) A monoclonal antibody that targets a NaV1.7 channel voltage sensor for pain and itch relief. *Cell* 157(6):1393–1404.
50. Clare JJ (2010) Targeting voltage-gated sodium channels for pain therapy. *Expert Opin Investig Drugs* 19(1):45–62.
51. Wang Y, et al. (2011) Merging structural motifs of functionalized amino acids and α -aminoamides results in novel anticonvulsant compounds with significant effects on slow and fast inactivation of voltage-gated sodium channels and in the treatment of neuropathic pain. *ACS Chem Neurosci* 2(6):317–322.
52. Zhou L, et al. (2010) First evidence of overlaps between HIV-Associated Dementia (HAD) and non-viral neurodegenerative diseases: Proteomic analysis of the frontal cortex from HIV+ patients with and without dementia. *Mol Neurodegener* 5:27.
53. Wilson SM, et al. (2012) Inhibition of transmitter release and attenuation of anti-retroviral-associated and tibial nerve injury-related painful peripheral neuropathy by novel synthetic Ca²⁺ channel peptides. *J Biol Chem* 287(42):35065–35077.
54. Fenteany G, et al. (1995) Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science* 268(5211):726–731.
55. Maruyama H, et al. (2004) Electrophysiological characterization of the tetrodotoxin-resistant Na⁺ channel, Na(V)1.9, in mouse dorsal root ganglion neurons. *Pflugers Arch* 449(1):76–87.
56. Davidson S, et al. (2014) Human sensory neurons: Membrane properties and sensitization by inflammatory mediators. *Pain* 155(9):1861–1870.
57. Joiner WJ, Khanna R, Schlichter LC, Kaczmarek LK (2001) Calmodulin regulates assembly and trafficking of SK4/IK1 Ca²⁺-activated K⁺ channels. *J Biol Chem* 276(41):37980–37985.
58. Brittain JM, et al. (2011) Neuroprotection against traumatic brain injury by a peptide derived from the collapsin response mediator protein 2 (CRMP2). *J Biol Chem* 286(43):37778–37792.
59. Chi XX, et al. (2009) Regulation of N-type voltage-gated calcium channels (Cav2.2) and transmitter release by collapsin response mediator protein-2 (CRMP-2) in sensory neurons. *J Cell Sci* 122(pt 23):4351–4362.
60. Weber AR, Schuermann D, Schär P (2014) Versatile recombinant SUMOylation system for the production of SUMO-modified protein. *PLoS One* 9(7):e102157.
61. Moutal A, et al. (2016) (S)-Lacosamide binding to Collapsin Response Mediator Protein 2 (CRMP2) regulates CaV2.2 activity by subverting its phosphorylation by Cdk5. *Mol Neurobiol* 53(3):1959–1976.