Characterization of NCS1-InsP3R1 interaction and its functional significance
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ABSTRACT
Inositol 1,4,5-trisphosphate receptors (InsP3Rs) are endoplasmic reticulum (ER)-localized channels that mediate Ca\(^{2+}\) release from the ER into the cytoplasm. We previously reported that an EF-hand Ca\(^{2+}\)-binding protein, neuronal calcium sensor 1 (NCS1), binds to the InsP3R and thereby increases channel open probability, an event associated with chemotherapy-induced peripheral neuropathy. However, the exact NCS1-binding site on InsP3R remains unknown. Using protein docking, co-immunoprecipitation, and blocking peptides, we mapped the NCS1-binding site to residues 66-110 on the suppressor domain of InsP3R type 1 (InsP3R1). We also identified Leu-89, a residue in the hydrophobic pocket of NCS1, as being critical for facilitating the NCS1-InsP3R1 interaction. Overexpression of WT NCS1 in MDA-MB-231 breast cancer cells increased Ca\(^{2+}\) signaling and survival, whereas overexpression of Leu-89 NCS1 variants decreased Ca\(^{2+}\) signaling and survival, further suggesting the importance of this residue in the NCS1-InsP3R1 interaction. In conclusion, we show that NCS1-InsP3R1 interaction enhances intracellular Ca\(^{2+}\) signaling in cells, and can be modulated by altering or occluding the hydrophobic pocket of NCS1. This improved understanding of the NCS1-InsP3R1 interaction may facilitate the development of management strategies for diseases resulting from aberrant NCS1 expression.

INTRODUCTION
As a universal second messenger involved in many signaling pathways, the intracellular calcium (Ca\(^{2+}\)) concentration is tightly regulated through a network of ion channels and pumps located on the plasma membrane and organelar membranes such as mitochondria and the endoplasmic reticulum (ER). Inositol 1,4,5-trisphosphate receptors (InsP3Rs) are ER-localized ion channels that regulate Ca\(^{2+}\) flow from the ER into the cytoplasm. InsP3Rs are activated by inositol 1,4,5-trisphosphate (InsP3), which is produced by a signaling cascade starting with ligands binding to various G protein-coupled receptors (GPCRs), resulting in the activation of phospholipase C (PLC), which then cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) to produce InsP3 (1). The InsP3Rs are involved in numerous cellular processes including apoptosis (2), secretion (3), and
memory and learning (4). Dysfunction of InsP3R-dependent signaling underlies a multitude of diseases ranging from cerebellar ataxia (5, 6) to bile duct obstruction (7). Similar to many other Ca\textsuperscript{2+} channels, the opening probability of the InsP3Rs is regulated by Ca\textsuperscript{2+}-binding proteins such as calmodulin (CaM) (8), Ca\textsuperscript{2+} binding protein 1 (CaBP1) (9), and neuronal calcium sensor 1 (NCS1) (10, 11), which allow for the fine tuning of Ca\textsuperscript{2+}-mediated Ca\textsuperscript{2+} signaling.

We previously showed that NCS1 binding to the InsP3R is a critical trigger for the development of chemotherapy-induced peripheral neuropathy (CIPN) (10, 12-14). NCS1 is a member of the neuronal calcium sensor (NCS) family, with high affinity and low capacity for Ca\textsuperscript{2+} binding (15). We showed that paclitaxel binds NCS1, which enhances NCS1 binding to the InsP3R, subsequently amplifying Ca\textsuperscript{2+} flux from the ER into the cytosol and causing pathological activation of calpain (10, 12-14). In another study, NCS1 was shown to physically and functionally interact with InsP3R1 and InsP3R2, and deletion of NCS1 decreased survival and contractile function in young mice (16). Similarly, NCS1 and InsP3R1 were shown to interact to regulate neurite outgrowth in chick dorsal ganglion cells (17). These studies suggest that the interaction between NCS1 and the InsP3Rs is important in many contexts and becomes dysregulated in pathological states. However, the exact binding site between NCS1 and the InsP3Rs has not yet been determined.

This study maps the exact interaction between NCS1 and InsP3R1, with the eventual aim of targeting this interaction for therapeutic treatments. We showed that NCS1 binds to residues 1-225 in the N-terminus of InsP3R type 1 (InsP3R1), a region also regulated by other Ca\textsuperscript{2+} sensors (8, 9). We also demonstrated that NCS1-InsP3R1 interaction is enhanced by Ca\textsuperscript{2+} in the physiologically relevant sub-μM range. Using competing polypeptides and mutagenesis, the important residues for the NCS1-InsP3R1 interaction was further restricted to residue Leucine-89 (L89) on NCS1, and Histidine-93 (H93) and Histidine-94 (H94) on InsP3R1. Blocking peptides corresponding to the region where InsP3R1 binds NCS1 region reduced the interaction between NCS1 and InsP3R1 (1-225) as well as full-length InsP3R1. The cell-penetrant version of one blocking peptide also reduced InsP3R1-dependent Ca\textsuperscript{2+} signaling in MDA-MB231 cells. To further investigate the functional relevance of L89 NCS1 variants, we found that overexpression of wild-type (WT) NCS1 increased InsP3R-dependent Ca\textsuperscript{2+} signaling, whereas overexpression of L89 NCS1 variants reduced Ca\textsuperscript{2+} signaling. Similarly, overexpression of WT NCS1 increased, whereas overexpression of L89 NCS1 variants decreased cell survival, further supporting the importance of residue L89 of NCS1. Overall, our findings expand the understanding of the biochemical components needed for regulation of Ca\textsuperscript{2+} homeostasis, as well as identify potential therapeutic targets for alleviating pathological conditions involving NCS1 and the InsP3Rs.

RESULTS

NCS1 binds the suppressor domain of InsP3R1. We previously showed that NCS1 bound full-length InsP3R1, and the interaction is enhanced by the addition of Ca\textsuperscript{2+} or paclitaxel (10, 12). As the addition of NCS1 to the cytoplasmic side of InsP3R1 increased channel activity (13), we hypothesized that NCS1 bound to a cytoplasmic region of InsP3R1. To narrow down the binding site, HA-tagged NCS1 and various fragments of InsP3R1 were co-expressed in HEK293 cells, followed by co-immunoprecipitation using NCS1 or HA antibodies (Fig. 1A-C). NCS1 co-immunoprecipitated the InsP3R fragment comprised of residues 1-346, but not other fragments, suggesting that NCS1 only binds to the suppressor region of InsP3R1 (Fig. 1B, 1C). Subsequently, we found that a GST-tagged fragment containing residues 1-225, which corresponded to the suppressor domain of InsP3R1, pulled down NCS1 (Fig. 1D). Similar to full-length InsP3R1, the interaction between NCS1 and InsP3R1 (1-225) was enhanced by the addition of Ca\textsuperscript{2+} (Fig. 1C) or
paclitaxel (Sup. Fig. 1). To determine the Ca\(^{2+}\) concentration relevant for NCS1-InsP3R1 interaction, we performed pulldown between GST-InsP3R1 (1-225) and NCS1 in Ca\(^{2+}\) concentrations ranging from 0 to 100 µM (Fig. 1D). The interaction was significantly enhanced by Ca\(^{2+}\) concentrations between 100 nM and 1 µM, which closely matches the Ca\(^{2+}\) binding curve of NCS1 (15). Using a log(agonist) vs. response fit from 4 experiments, the EC50 of the interaction was estimated to be -6.8, which corresponded to a Ca\(^{2+}\) concentration of 160 nM (Fig.1E).

**L89 residue of NCS1 facilitates NCS1-InsP3R1 Ca\(^{2+}\)-dependent interaction.** To experimentally identify the predicted binding site, we performed pulldown experiments between GST-tagged InsP3R1 (1-225) and NCS1 variants. These variants of NCS1 have mutations in pairs of hydrophobic residues that span the hydrophobic cleft and are not located within the Ca\(^{2+}\)-binding motifs (Fig. 2A). Binding between InsP3R1 (1-225) and NCS1 variants F48E_W103K, Y108E_M121K, or F169E_M156K was enhanced by Ca\(^{2+}\), similar to WT NCS1 (Fig. 1B). However, NCS1 mutant L89E_W103K showed an opposite trend, where the addition of Ca\(^{2+}\) almost completely inhibited its binding to InsP3R1 (1-225). Additional experiments demonstrated that the W103K mutant binding to InsP3R1 (1-225) was comparable to WT NCS1. In contrast, when residue L89 alone was mutated to A, E, or K, binding to InsP3R1 (1-225) in Ca\(^{2+}\)-containing solutions was reduced with the largest deficit observed for the L89K mutant (Fig. 2B). Pulldown experiments between GST-tagged InsP3R1 (1-225) and WT, L89A, or L89K NCS1 at increasing Ca\(^{2+}\) concentrations also demonstrated the opposite binding trend between WT and L89 NCS1 variants to InsP3R1 (1-225) (Fig. 2C, 2D). To rule out the possibility that the reduced binding efficiency was due to the Ca\(^{2+}\) binding property of the variants, isothermal titration calorimetry measurements were performed and showed that the L89K mutant bound Ca\(^{2+}\) similarly to WT NCS1 (Supp. Fig. 2).

**Docking analysis.** After identifying InsP3R1 (1-225) as the NCS1-binding region, we took advantage of the available crystal structures of full-length NCS1 and the N-terminal fragments of InsP3R1 to perform computational docking to predict the binding site. Docking was performed between full-length, Ca\(^{2+}\)-bound NCS1 (PDB=1g8i, chain A) and 2 fragments of InsP3R1: 1-246 (PDB=1XZZ) and (1-604) (PDB=3UJ4, chain A) using the programs Zdock and Cluster Pro. Top docking results indicated that the helical arm of InsP3R1 (1-225), which encompasses residues 66-110, interacts with the hydrophobic pocket of NCS1 (Fig. 3A). Docking results also supported our observations that L89 is important for binding to InsP3R1 (Fig. 3B). L89 appears to make direct contact with residues H93 and H94 of InsP3R1 and forms additional hydrophobic contacts. We confirmed this by showing that the H93A_H94A InsP3R1 variant showed decreased binding to NCS1 in the presence of Ca\(^{2+}\) (Fig. 3C). Similar results were obtained using Cluster Pro, which utilized a different docking algorithm (Fig. 3D).

**InsP3R1 peptides corresponding to NCS1 binding region reduce NCS1-InsP3R1 interaction.** As our docking model predicted that the region spanning from residues 66-110 of InsP3R1 interacted with NCS1, we tested this prediction by adding in polypeptides spanning the region (peptides 68-80, 82-96, 84-98 (Fig. 4A)). The peptides were similar regarding charge, hydrophobic content, and length. Both peptides (68-80) and (82-96) reduced the interaction between NCS1 and InsP3R1 (1-225) (Fig. 4B), whereas another peptide (84-98) did not (Fig. 4B, 4C). To further validate this, we repeated co-immunoprecipitation experiments between NCS1 and full-length InsP3R1 from mouse cerebellum lysate. The peptide (82-96) reduced this interaction (Fig. 4D, 4E), further supporting the finding that this region of InsP3R1 is important for the binding between NCS1 and InsP3R1.

**Cell-penetrant TAT-(68-80) peptide decreases InsP3R-dependent Ca\(^{2+}\)signaling.** To test whether InsP3R1 blocking
peptides can block the interaction in vivo and lead to a functional difference, we treated MDA-MB231 cells with 20 μM cell-penetrant TAT-(68-80), or the TAT-scrambled control, 45 minutes prior to Ca\(^{2+}\) imaging. To rule out Ca\(^{2+}\) flux from the extracellular environment, imaging was carried out in Ca\(^{2+}\) free buffer and with uridine triphosphate (UTP), a specific P2Y GPCR agonist that leads to the production of InsP3 (18). Compared to cells treated with the scrambled control, cells treated with TAT-(68-80) showed similar rate of response (Fig. 5B), but lower maximum amplitude (Fig. 5C), area under the curve (Fig. 5D), and rate of rise (Fig. 5E) of response. These observations suggested that TAT-(68-80) could block NCS1-InsP3R1 in vivo to decrease Ca\(^{2+}\) signaling.

**WT NCS1 increases, whereas L89 NCS1 variants decrease, Ca\(^{2+}\) signaling.** As L89 NCS1 variants had diminished binding to InsP3R1, we predicted that L89 NCS1 variants would be loss-of-function variants. To test this hypothesis, we overexpressed WT NCS1 and NCS1 variants in MDA-MB231 cells, a human breast cancer cell line in which we previously showed that overexpression of WT NCS1 led to increased cell survival and metastasis [27, 28]. Monoclonal cell populations were generated in which NCS1 levels in overexpressing cells were severalfold more than the native level (Supp. Fig 3A). Previous studies reported that overexpression of WT NCS1 increases, whereas reducing NCS1 decreases, InsP3R1-dependent Ca\(^{2+}\) signaling in several cell types (10, 16, 18, 19). Because L89 NCS1 variants do not bind InsP3R1 efficiently, we predicted that overexpressing these variants would not increase Ca\(^{2+}\) signals, or even decrease signaling. As expected, cells overexpressing WT NCS1 showed increased Ca\(^{2+}\) signaling, whereas cells overexpressing L89A and L89K NCS1 showed unchanged or decreased Ca\(^{2+}\) signaling (Fig. 6A). The percentage of cells responding to 3 μM UTP was comparable across the different cells tested (Fig. 6B). Compared to the control cells, cells overexpressing WT NCS1 had an increased maximum amplitude (Fig. 6C), rate of rise (Fig. 6D) and area under the curve (Fig. 6E). In contrast, cells overexpressing L89 NCS1 variants showed unchanged decreased maximum amplitude, rate of rise, and area under the curve. As both WT NCS1 and L89K NCS1 bind Ca\(^{2+}\), the reduction in Ca\(^{2+}\) signaling is likely not due to altered Ca\(^{2+}\) buffering, but rather due to the reduction in the NCS1-InsP3R1 interaction. Ca\(^{2+}\) signals following the addition of 1 μM of the ionophore ionomycin were similar in the different cell types, suggesting that intracellular Ca\(^{2+}\) loading was similar among the different cells (Supp. Fig. 4)

**WT NCS1 increases, whereas L89 variants decrease, survival.** NCS1 was previously shown to increase survival of neuronal SH-SY5Y cells (20), cortical neurons (21) and MDA-MB231 (22, 23), partly through upregulating the pAKT pathway. As we observed that the L89 NCS1 variants decreased Ca\(^{2+}\) signaling, we hypothesized that overexpression of L89A would also decrease cell survival. Using a colony formation assay to measure cell survival, we found a significant difference between cells expressing WT or mutant NCS1 as compared to control cells (Fig. 7). When 100 cells were plated initially, cells that overexpressed WT NCS1 covered 4.5-fold more area than CTRL cells expressing the empty vector. As expected, cells that overexpressed L89A NCS1 covered 75% reduction in the area covered as compared to the control (Fig. 7B). Similar results were observed when 500 cells were initially plated (Fig. 7C). There was no significant difference in the proliferation rate among CTRL, WT NCS1 overexpression (OE) and L89K NCS1 OE cells (Supp. Fig. 5), suggesting that the difference observed in the colony formation assay was primarily caused by differences in survival rate.

**DISCUSSION**

Ca\(^{2+}\) is a universal second messenger that regulates diverse processes including neurotransmitter release, muscle contraction, and cell death. As such, cytosolic Ca\(^{2+}\) is tightly


regulated, partly through the controlled release of Ca\textsuperscript{2+} from ER stores via InsP3Rs. One of the mechanisms used to regulate InsP3Rs is the binding of members of the EF-hand Ca\textsuperscript{2+}-binding proteins, specifically, CaM, CaBP1, and NCS1. InsP3R1 contains three CaM bindings sites, where two sites are in the suppressor domain (residues 1-225), and one site is in the modulatory domain (residues 1564-1585) (24). Binding of CaM to the suppressor domain decreases Ca\textsuperscript{2+} release (8), and binding of CaM to the modulatory domain does not affect Ca\textsuperscript{2+} release (24). There is less consensus concerning the effect of CaBP1 binding to the suppressor domain, where both inhibitory and activating effects have been reported (9, 25). We found that NCS1 binds only in the suppressor domain and NCS1 binding is stimulatory (13). Each of these interactions between a Ca\textsuperscript{2+} binding protein and the InsP3R shows that regulating Ca\textsuperscript{2+} release through InsP3R1 channel is central to regulating cellular processes.

**Physiological significance of NCS1-InsP3R interaction.** NCS1 is a low-capacity, high affinity Ca\textsuperscript{2+} binding protein. Although NCS1, CaM, and CaBP1 share <50% homology at the primary amino acid sequence, they are similar at the secondary and tertiary structure with 4 EF-hands. NCS1, CaM, and CaBP1 also bind to and regulate similar target proteins (26). We propose that regulation of the same protein by similar Ca\textsuperscript{2+}-binding proteins may be essential in allowing the fine-tuning of a cell’s response to fluctuation in Ca\textsuperscript{2+} levels. For example, NCS1 is sensitive to Ca\textsuperscript{2+} changes from 10 nM to 300 nM, whereas CaM is sensitive to Ca\textsuperscript{2+} changes from 100 nM to 100 uM (15). Our previous work shows that binding of NCS1 to InsP3R1 increases the open probability of InsP3R1, leading to a larger Ca\textsuperscript{2+} release (13). A further study may investigate possible interactions among NCS1, CaM, and CaBP1 for the suppressor domain of InsP3R1.

Although our study focuses on the interaction between NCS1 and InsP3R1, we propose that NCS1 also interacts with other subtypes of InsP3R, namely types 2 and 3. In fact, NCS1 was shown to interact with InsP3R2 in heart (16). Our preliminary docking results of NCS1 to the suppressor domain of InsP3R3 (Supp. Fig. 6) suggest that the binding is similar. The suppressor domains of the three subtypes of InsP3Rs are highly homologous, sharing more than 77% identity and 93% similarity. In comparison, the adjacent regulatory domains of InsP3Rs share 67% identity and 88% similarity. Residue H94 is conserved across all three subtypes, whereas H93 is changed to Q in types 2 and 3 (Supp. Fig. 7). Whether this difference affects the binding affinity of NCS1 to types 2 and 3 remains to be investigated.

**Identification of specific binding regions that can facilitate rational drug design.** We showed that L89 NCS1 variants displayed an opposite trend to WT NCS1 in which Ca\textsuperscript{2+} almost completely inhibited the binding to InsP3R1 (1-225). In our docking model, L89 is close to the helical arm of the suppressor domain of the InsP3R1, specifically to H93 (Fig. 3B). The importance of the L89 NCS1 residue was further supported where L89 NCS1 variants reduced instead of increased Ca\textsuperscript{2+} signaling. Previous work (27) also showed that human L89A NCS1 failed to rescue memory deficits in ncs1-null C. elegans, further suggesting the functional significance of this residue. The identification of the L89 NCS1 residue as critical for NCS1-InsP3R1 binding can allow for drug screening focusing on the residue. A similar strategy was successful for blocking the interaction between NCS1 and the RIC8 Guanine Nucleotide Exchange Factor A (RIC8A), supporting this as a feasible approach (28).

**Cell-penetrant peptides for modulating NCS1-InsP3R interaction.** We showed that peptides consisting of residues 68-80 and 82-96 of the InsP3R could reduce the interaction between NCS1 and InsP3R1. Cell-penetrant TAT-(68-80) could also decrease InsP3R-dependent Ca\textsuperscript{2+}-signaling 45 minutes after treatment. Interestingly, a D2R peptide used in a previous study (29) to reduce NCS1-D2R interaction increased NCS1-InsP3R1 interaction (Sup. Fig.8). We hypothesize that the D2R peptide stabilizes the hydrophobic pocket of NCS1 to enhance NCS1-InsP3R1 interactions.
interaction. Therefore, another approach would be to use these peptides in combination as scaffolds for producing small compounds that could moderate the interaction both up or down. For example, cell-penetrant peptides can be tested in animal models to test if CIPN can be alleviated.

A limitation in our study is that we cannot decisively rule out whether other NCS1- or InsP3R1-protein partners also play a role in the functional differences observed. As NCS1 is a multifunctional protein with multiple known interacting partners, L89 NCS1 variants may also exhibit altered interaction with these partners. Similarly, the N-terminus of InsP3R is regulated by various Ca\(^{2+}\) sensors, and a blocking peptide corresponding to this region may also block InsP3R interaction with these regulatory proteins. However, as modification of NCS1-InsP3R interaction through targeting either NCS1 or InsP3R1 resulted in similar changes, including decreased Ca\(^{2+}\) signaling, we are confident that NCS1-InsP3R1 interaction, at minimum, contributed to the differences observed.

**Conclusion.** Here, we show that NCS1 binds to the suppressor domain of InsP3R and that this binding is enhanced by the addition of Ca\(^{2+}\). We also identified one residue of NCS1 (L89) and residues H93 and H94 of InsP3R1 as important for the interaction. Mutations to L89 of NCS1 or peptides corresponding to the binding region of InsP3R1 reduced the interaction. To test if NCS1-InsP3R1 binding was functionally relevant, we showed that changes in the interaction, either through a cell-penetrant peptide or through overexpressing L89 NCS1 variants, modulated Ca\(^{2+}\) signaling. Further studies can examine how blocking the NCS1-InsP3R interaction, either by identification of novel drugs that inhibit binding or modifications of peptides centered on the relevant residues of NCS1 or InsP3R, can be used as therapeutic strategies.

**MATERIALS AND METHODS**

**Mammalian NCS1/IP3R1 co-immunoprecipitation.** Transfected HEK293 cell lysate expressing InsP3R1 fragments and HA-tagged NCS1 were diluted to obtain 500 μL of 0.4 μg/μL per sample. Each sample was incubated with 10 μL of anti-NCS1 antibody (FL190, Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4°C, followed by incubation with 30 μL of protein-A magnetic beads (PureProteome, Millipore Sigma, Waltham, MA, USA) for 1 h at 4°C. Beads were washed 3 times with cold phosphate buffered saline (PBS) (American Bio, Canton, MA, USA), and then eluted and protein levels were quantified using Western blot analysis.

**Western Blot analysis.** Western blots were performed using the NuPAGE system (Thermo Fisher Scientific, Waltham, MA, USA) and PVDF membrane with the Bio-Rad wet transfer system (Bio-Rad Laboratories, Santa Rosa, CA, USA). Primary antibodies used were anti-NCS1 FL-190, anti-HA (Santa Cruz Biotechnology, Dallas, TX, USA), anti-FLAG (Sigma-Aldrich, Waltham, MA, USA), T1NH (1:1000, custom antibody that binds N-term of manufacturer's instructions unless otherwise stated. Vectors for mammalian expression of full-length and fragments of InsP3R1 were gifts from Dr. David Yule (University of Rochester Medical Center). The vector for HA-tagged NCS1 was previously made in the lab. HEK293 cells (American Type Culture Collection, Manassas, VA, USA) were co-transfected with 1 ug each of InsP3R1 and HA-tagged NCS1 using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Cells were harvested 48 h after transfection, lysed using M-PER mammalian protein extraction reagent (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with protease inhibitor (P2714, Sigma Aldrich, Burlington, MA, USA), and centrifuged at 13,000 rpm for 15 min at 4°C. The pellet was discarded and the lysate was collected. Protein concentration was determined using Pierce BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA). Cell lysate was stored at -20°C until further use.
InsP3R1, YU272 (1:1000, custom antibody that binds C-term of InsP3R1). Western blot was performed and the results quantified as previous described (10).

Bacterial NCS1 and GST-IP3R1 (1-225) protein expression and purification. pGEX6p2 vector carrying the coding sequence for InsP3R1 (1-225) was a gift from Dr. Jan Parys (KU Leuven). pET-21 vector carrying the coding sequence for NCS1 was prepared in the lab previously. Each plasmid was transformed into BL21-CodonPlus (DE3)-RIPL competent cells (Agilent Technologies, Santa Clara, CA, USA). Single clones were grown and crude bacterial lysate was prepared as previously described (8, 30).

Preparing NCS1 mutant plasmid. Plasmids encoding wild-type NCS1 for protein expression (pET-21/NCS-) or in pcDNA3.1 for transfection were described previously (31). Plasmids encoding NCS1 variants (E120Q) were prepared by site-directed mutagenesis using a Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Primers were designed using the company’s website. Plasmid sequences were confirmed by automated DNA sequencing. NCS1 expressed in bacteria was not tagged. For mammalian expression, a C-terminal tag was introduced using the reverse primer. WT, L89A, L89K were tagged with HA, FLAG, and His6 respectively, and E120Q NCS1 was untagged.

Monitoring calcium-dependency. To monitor the calcium dependence of NCS1/InsP3R interactions, the EGTA concentration was fixed at 1 mM. To achieve different concentrations of free Ca2+, the amount of total CaCl2 needed was calculated using the Ca-EGTA calculator (http://maxchelator.stanford.edu/CaEGTA-TS.htm) with T = 21.5 °C, pH = 7.4, N = 0.163 and EGTA = 1 mM to obtain free Ca2+ concentration ranging from 10⁻⁸ to 10⁻³ M. Appropriate Ca2⁺ concentrations were maintained in all working solutions for each condition.

For GST pulldown, glutathione sepharose 4B (GE Healthcare, Waukesha, WI, USA) was used according to manufacturer’s instruction. 30 uL of beads were washed 3 x 1mL with washing buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EGTA and a predetermined concentration of Ca²⁺), and then incubated with 500 ug of crude GST-IP3R1 lysate for 1 h with gentle end-over-end rotation at room temperature. Subsequently, unbound proteins were removed and the beads were washed with 3 x 1 mL washing buffer. Next, the beads were incubated with 10 nM NCS1 in washing buffer for 2 h at room temperature. Unbound proteins were removed and the beads were washed with 3 x 1 mL washing buffer. Bound proteins were then eluted with 20 uL of 20 mM glutathione (AmericanBio, Canton, MA, USA) in 50 mM Tris, pH 7.4.

Docking. The structural basis for interactions between NCS1 and InsP3R were independently predicted using the protein-protein docking programs ZDOCK (32, 33) and Cluster Pro 2.0 (34). In both ZDOCK and Cluster Pro, the structure of full-length calcium bound NCS1 (PDB 1G8I) was input as the ‘ligand’; and the crystal structure of InsP3R containing residues 1-604 (PDB 3UJ0) was input as the ‘receptor’. The structure of InsP3R used for docking contains both the suppressor domain (1-223) and the IP₃ binding core (224–579). The top-ranked complexes from ZDOCK (ZDOCK score = 1694) and Cluster Pro were chosen for analysis in the macromolecular visualization program PyMOL (version 0.99 Schrödinger, LLC).

Peptides. Synthetic peptides were purchased from Genscript (USA) and delivered with >95% purity, with TFA removal. Peptide sequences, solvents and concentrations were detailed in Table 1.

| Label | Sequence | Stock concentration |
|-------|----------|---------------------|
| (68-80) | AQKQFWKAALKPGA | 1 mM in PBS with 10% DMSO |
| (82-96) | TTDAVLNLKLLHAAD | |

Table 1: Peptides information
Cell culture and generation of stable cells. MDA-MB231 cells (American Type Culture Collection, Manassas, VA, USA) were maintained at 37°C, 5% CO₂, in DMEM supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin. Stable MDA-MB231 cells were generated through transfection with a control vector or vectors for overexpression of WT and mutant NCS1 using lipofectamine. 2 days after selection, stable cells were selected with 2 mg/mL G418 (American Bio) for two weeks and the overexpression was confirmed using Western blot analysis. Single clones were selected and expanded for a homogeneous population. Stable cells were maintained in the same medium supplemented with 1 mg/mL G418.

Calcium imaging. 22x22 mm glass coverslips were washed with 70% ethanol, rinsed 3 times with MilliQ water and left to dry and sterilized under UV in a cell culture hood. 1x10⁵ cells were plated on each coverslip 1 day before imaging. HEPES-buffered saline (140 mM NaCl, 1.13 mM MgCl₂, 4.7 mM KCl, 2 mM CaCl₂, 10 mM D-glucose, and 10 mM HEPES, adjusted to pH 7.4 with NaOH) was used for preparing Ca²⁺ dye solution and for imaging. For Ca²⁺-free HEPES buffer, CaCl₂ was replaced with MgCl₂ and 0.1 mM EGTA was added to chelate Ca²⁺. 4 μM Fluo-4-am dye was prepared by dissolving powder Fluo-4-am (Thermo Fisher Scientific, Waltham, MA, USA) in Ca²⁺-containing HEPES buffer supplemented with 0.03% Pluronic acid (Thermo Fisher Scientific, Waltham, MA, USA). On the day of imaging, each coverslip was incubated in 4 uM Fluo-4-am for 45 min at 37°C. Cells were then washed 3 times and bathed in HEPES-buffered saline solution for 5 min before Ca²⁺ measurements began. The signal for each cell was subtracted by the background signal and normalized to the first 10 seconds baseline. Cells that showed a UTP response of greater than or equal to 1.1-fold of baseline were considered to be responding and included in the data analysis. Rate of rise was estimated as the gradient between 25% and 75% maximum amplitude. All experiments were conducted at room temperature. All figures depicting Ca²⁺ imaging traces are the average of all responding cells in 9-21 coverslips from at least 3 independent recordings.

Colony formation assay. For colony formation assays, once cells in a T75 flask reach confluence of 80-90%, they were detached by the addition of 2 mL TrypLE (Thermo Fisher Scientific, Waltham, MA, USA), followed by dilution in 5 mL of fresh medium. Cell density was determined using a hemocytometer. Subsequently, a total number of 100 or 500 cells was added to each well of a 12-well Corning plate (Thermo Fisher Scientific, Waltham, MA, USA). Cells were then left undisturbed in the incubator over 14 days. After 14 days, colonies were fixed and stained with 2.5% crystal violet solution, washed to remove excess dye and scanned with a conventional scanner. The total area covered was determined with ImageJ using a published plugin (35). Data were obtained from 3 independent experiments and were represented as total area covered in every individual well normalized to controls.

Data analysis. Data management and calculations were performed using PRISM Statistical Software 7. For comparison between two groups, unpaired, two-tailed student t-test was carried out. For comparison of more than two groups, one-way analysis of variance (ANOVA) followed by Dunnet’s multiple comparisons test was carried out to determine if the mean from one group is statistically different from that of another group.
A p-value < 0.05 was considered to be statistically significant and the following notations were used in all figures: * for p<0.05, ** for p<0.01, *** for p<0.005, and **** for p<0.0001. All error bars shown are standard deviation unless otherwise stated.

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Competing interests. B.E.E is a founder of Osmol Therapeutics, a company that is targeting NCS1 for therapeutic purposes.

Data and materials. Data and materials are available upon request.
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**ABBREVIATIONS AND NOMENCLATURE**

ER: endoplasmic reticulum
InsP3R: Inositol 1,4,5-trisphosphate receptor
NCS1: Neuronal calcium sensor 1
MDA-MB231: an epithelial mammary breast cell line derived from metastatic site
GPCR: G protein-coupled receptor
Figure 1. NCS1 binds the N-terminus of InsP3R1 in a Ca\textsuperscript{2+} dependent manner. (A) Diagram showing the InsP3R1 fragments tested in co-IP experiments using NCS1 antibody and HEK293 cell lysates co-expressing fragments of InsP3R1 and HA-tagged NCS1. A check shows fragments that pulled down NCS1, a cross shows fragments that did not pull down NCS1. (B) Representative results with fragments F1, F2, F3 and F4. All experiments were repeated at least 3 times with similar results. (C) F1 was also co-IPed along with HA-tagged NCS1 using HA antibody. (D) A representative plot showed that NCS1 binding to InsP3R1-(1-225) (F6) was enhanced by Ca\textsuperscript{2+} concentrations above 1 μM. (E) Quantification from 4 pulldown experiments at different free Ca\textsuperscript{2+} concentrations. An estimation using the EC50 value from a log(agonist) vs. response fit gave a value of 160 nM. Error bars shown were standard error of measurement (SEM).
A

Free Ca^{2+} concentration

IB: NCS1 20 kDa

B

Free Ca^{2+} concentration

IB: NCS1 20 kDa

C

Free Ca^{2+} concentration

WT NCS1

L89A NCS1

L89K NCS1

D

Pulldown with varying [Ca^{2+}]

Normalized to 0 Ca^{2+}

Log [Free Ca^{2+}]
Figure 2. The L89 residue on NCS1 is important for the Ca\(^{2+}\)-dependent NCS1-InsP3R1 interaction. (A) GST-InsP3R1 (1-225) pulldown of WT and mutant NCS1 in Ca\(^{2+}\)-free and Ca\(^{2+}\)-containing conditions. Only the L89E_W103K mutant showed an inverted trend where Ca\(^{2+}\) reduced instead of increased interaction. (B) Mutation of the L89 residue to A, E or K all decreased the NCS1-InsP3R1 interaction in the presence of Ca\(^{2+}\). (C) Interaction between the WT and L89 NCS1 variants over a range of physiologically relevant Ca\(^{2+}\) concentrations from 0 to 10 μM. (D) Quantification of 4 experiments for WT NCS1 (same data as shown in 1E), 4 experiments for L89K and 3 experiments for L89A. * denoted statistically significant difference between WT NCS1 and L89 NCS1 variants at that specific Ca\(^{2+}\) concentration (Student t-test, adjusted for multiple comparison). Fit show was from log (agonist) vs. response, error bars shown were SEM.
Figure 3. (A) Prediction of the molecular interaction between Ca\(^{2+}\)-bound NCS1 (white, PDB 1G8I) and a fragment of InsP3R1 (blue) containing residues 1-604 (PDB 3UJ0) using the ZDOCK server. (B) L89 (shown in pink) of NCS1 forms interactions with the arm motif of the suppressor domain of InsP3R1 (residues 66-110). Mutations L89A, L89E and L89K would likely disrupt binding to this region of the InsP3R. (C) Mutation of H93_H94 to A reduced NCS1-InsP3R1 interaction in the presence of Ca\(^{2+}\). (D) A nearly identical binding geometry for complex formation between NCS1 and InsP3R1 was predicted by the ClusPro server using an independent docking algorithm. (E) Visualization of NCS1 binding to full length InsP3R. Molecular interactions between NCS1 (white, PDB 1G8I) and a fragment of InsP3R1 (blue) containing residues 1-604 (PDB 3UJ0) were predicted using the ZDOCK server and superimposed in PyMol on the structure of full-length InsP3R1. The predicted binding site is in the cytoplasmic domain and appears accessible by NCS1.
Figure 4. InsP3R1 peptides corresponding to NCS1 binding region reduced NCS1-InsP3R1 interaction. (A) Diagram showing the InsP3R1 peptides tested within the predicted binding region spanning residues 66-110, with the peptides used shown in boxes. (B) Representative blot of pulldown between GST-InsP3R1 (1-225) and NCS1 in the presence of various concentrations of peptides and 10 μM Ca^{2+}. (C) Quantification of 3 independent experiments with peptides added at 20X molar excess (2 μM peptide, 100 nM NCS1). Addition of peptides (68-80) and (82-96) resulted in a 58% (p = .0007) and 16% reduction (p = 0.02) in the amount of NCS1 pulled down respectively, whereas addition of peptide (84-98) did not change the amount of NCS1 pulldown
(p = 0.09) (two-tailed student t-test comparing the no peptide control with the specific peptide) (D) Peptide (82-96) also reduced the interaction between NCS1 and full-length InsP3R1 by 60% in mouse cerebellum lysate (n = 3, two-tailed student t-test, p = 0.002).
Figure 5. Cell-penetrant TAT-(68-80) decreased InsP3R-dependent Ca\textsuperscript{2+} signaling. (A) Combined traces of all coverslips (n = 12 for each treatment) for MB231 cells treated with either 20 μM TAT-(68-80) or TAT-scrambled control (TAT-SCRB). UTP was added from 10s to 40s. (B) The number of cells responding per coverslip were similar across different cell types (p = 0.68). Compared to the scrambled control, cells treated with TAT-(68-80) showed decreased (C)
maximum amplitude (p = 0.014), (D) area under the curve (p = 0.036) and (E) rate of rise (p = 0.04). Statistical analysis was carried out using unpaired two-tailed student t-test.
A 3 μM UTP in Ca\(^{2+}\) free buffer

B Percentage of cells responding

C Max UTP amplitude

D Area under the curve for UTP response

E Rate of rise

*** n/s *
Figure 6. L89 NCS1 variants did not change or decreased InsP3R1-dependent Ca\(^{2+}\) signaling. (A) Combined traces of all coverslips for different cell types (n = 21 coverslips for empty vector control, 15 for WT NCS1, 15 for L89A NCS1 and 11 for L89K NCS1). UTP was added from 10s to 30s. (B) The number of cells responding per coverslip were similar across different cell types (one-way ANOVA, p = 0.22). Compared to control, cells overexpressing WT NCS1 showed increased (C) maximum amplitude, (D) area under the curve and (E) rate of rise. Cells overexpressing L89A, L89K NCS1 showed either similar or decreased (C) maximum amplitude, (D) rate of rise and (E) area under the curve. One-way ordinary ANOVA was carried out, followed by post-hoc Dunnett's multiple comparison test with * for p<0.05, ** for p<0.01 and *** for p<0.005.
Figure 7. L89 NCS1 variants decreased survival. (A) Representative image for colony formation assay. (B) When 100 cells were initially plated, cells overexpressing WT NCS1 showed a significant increase in survival (p = 0.0001). In contrast, cells overexpressing L89A NCS1 showed a significant decrease in survival (p = 0.028). Cell overexpressing L89K NCS1 also showed a reduction in survival, although the reduction was not statistically significant (p = 0.44). (C) Similar results were obtained when 500 cells were initially plated, and the difference between CTRL and L89K cells was statistically significant (p = 0.035). Statistical analysis was performed on 4 independent experiments for each cell type, with duplicates or triplicates for each experiment. One-way ordinary ANOVA was carried out, followed by post-hoc Dunnett’s multiple comparison test with * for p<0.05, ** for p<0.01 and *** for p<0.005.
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