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Molecular basis for the binding and selective dephosphorylation of Na\(^+\)/H\(^+\) exchanger 1 by calcineurin

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Very little is known about how Ser/Thr protein phosphatases specifically recruit and dephosphorylate substrates. Here, we identify how the Na\(^+\)/H\(^+\)-exchanger 1 (NHE1), a key regulator of cellular pH homeostasis, is regulated by the Ser/Thr phosphatase calcineurin (CN). NHE1 activity is increased by phosphorylation of NHE1 residue T779, which is specifically dephosphorylated by CN. While it is known that Ser/Thr protein phosphatases prefer pThr over pSer, we show that this preference is not key to this exquisite CN selectivity. Rather, a combination of molecular mechanisms, including recognition motifs, dynamic charge-charge interactions and a substrate interaction pocket lead to selective dephosphorylation of pT779. Our data identify T779 as a site regulating NHE1-mediated cellular acid extrusion and provides a molecular understanding of NHE1 substrate selection by CN, specifically, and how phosphatases recruit specific substrates, generally.
The plasma membrane Na\(^+\)/H\(^+\) exchanger 1 (NHE1, SLC9A1) is a central regulator of cellular pH and volume, and thus is important for cell proliferation, survival, and motility in mammalian tissues. As a consequence, its dysregulation leads to disease, especially cancer and cardiovascular disorders\(^1\)\(^3\). NHE1 comprises a 12 transmembrane-helix domain that mediates Na\(^+\)/H\(^+\) exchange and a C-terminal intracellular domain that is heavily phosphorylated and functions as a protein:protein interaction hub. Critically, these modifications and interactions control NHE1 activity. The C-terminal ~135 residues of NHE1 (NHE1ct; residues I680-Q815) is an intrinsically disordered region (IDR) that contains most of the identified NHE1 phosphorylation sites\(^4\)\(^5\).

Ser/Thr kinases use protein:protein interactions and sequence-specific recognition sequences to identify and phosphorylate their substrates; indeed, for this reason >10,000 kinase phosphorylation sites have been identified, allowing the functional importance of these sites to be readily investigated. Multiple mechanisms regulate NHE1 activity including increases in the free, intracellular Ca\(^{2+}\) concentration (\([Ca^{2+}]_i\))\(^6\)\(^7\), and phosphorylation by the MAP kinase, ERK2/1, p38, and c-Jun N-terminal kinase (JNK)\(^8\)\(^9\). We recently discovered that ERK2-mediated NHE1 phosphorylation is achieved, in part, via direct binding between ERK2 and the NHE1ct, resulting in ERK2-mediated phosphorylation of six Ser/Thr residues that conform to the ERK2 recognition sequence: [S/T]P\(^1\)\(^1\).

Much less is known about the specificity controlling the reversing dephosphorylation reactions, which is reflected by the fact that only ~400 phosphatase:substrate pairs are currently known. This is due, in part, to an apparent lack of phosphatase-specific recognition sequences, making it impossible to use bioinformatics to understand the substrate:enzyme relationship\(^1\)\(^2\). NHE1 interacts with, and is likely regulated by, protein phosphatase 1\(^1\)\(^3\) and 2A\(^1\)\(^4\) and the Tyr phosphatase SHP2\(^1\)\(^5\). A direct interaction of NHE1 with calcineurin (CN, protein phosphatase 2B or PP3) was also demonstrated\(^1\)\(^6\). However, if NHE1 is a direct substrate of any of these phosphatases, which residues are dephosphorylated and if this regulation has consequences for NHE1 function is still unknown.

CN is a ubiquitously expressed Ca\(^{2+}\)-dependent Ser/Thr phosphatase\(^1\)\(^7\). It regulates multiple physiological processes including development, cardiac function, and the immune response\(^1\)\(^8\). CN is a heterodimer composed of calcineurin A (CNA) and B (CNB), where CNA can be further divided into four functional domains: the catalytic domain, the CNB-binding domain, a calmodulin (CaM)-binding domain, and an autoinhibitory domain (AID), which blocks the CN active site\(^1\)\(^9\)\(^,\)\(^2\)\(^0\). Upon an increase in [Ca\(^{2+}\)]\(_i\), both CNB and CaM become Ca\(^{2+}\)-loaded, ultimately displacing the AID from the catalytic site, stimulating phosphatase activity. However, how CN recognizes its substrates is only poorly understood.

Recently, it was shown that CN recruits regulators, inhibitors, and substrates using two short linear motifs (SLiMs), the PxxIxIT and the LxVP motifs\(^1\)\(^2\)\(^1\). These SLiMs are typically found in intrinsically disordered proteins/regions (IDPs/IDRs), and bind to the corresponding PxxIxIT and LxVP-binding pockets in CN. The PxxIxIT motif binds the catalytic domain of CNA\(^2\)\(^2\), whereas the LxVP motif binds to a hydrophobic cleft at the interface of the CNA and CNB subunits\(^2\)\(^1\). In contrast to the PxxIxIT-binding pocket, the binding cleft for the LxVP motif is only accessible in the active conformation of CN\(^2\)\(^3\). The interaction between NHE1 and CN was shown to depend on a PxxIxIT motif in the NHE1ct\(^1\)\(^6\). However, it is not known if NHE1ct also has an LxVP motif. Further, if and how these motifs influence substrate recognition and specificity, or if there are other features that define substrate specificity is currently unknown for CN specifically and for Ser/Thr phosphatases generally.

Here, we integrate multiple complementary molecular and cellular experiments to show how CN is recruited to NHE1. Furthermore, we identify an NHE1 phosphorylation site, T779, that is critical for regulating NHE1 transport activity. Further, we show that CN specifically dephosphorylates pT779 to return NHE1 activity to basal levels. Finally, using molecular and cellular experiments, we define the key interactions that generate this CN selectivity at the CN active site. Together, these data not only reveal a key role for phosphatases in controlling NHE1 activity, but also provide molecular insights into the multiple mechanisms used by CN to achieve substrate specificity.

**Results**

**NHE1 binds CN using a PxxIxIT and an LxVP motif.** NHE1 was previously shown to bind CN using a PxxIxIT motif, T\(^7\)\(^1\)PVTITD\(^2\)\(^2\)\(^0\)\(^1\)\(^6\). This sequence is present in the intrinsically disordered C-terminal tail of NHE1 (residues I680-Q815, hereafter referred to as NHE1ct; Fig. 1a; variants used in this study are summarized in Supplementary Fig. 1; CN overview is summarized in Fig. 1b)\(^5\). An alignment of 15 diverse NHE1ct sequences from multiple species revealed the presence of a putative LxVP motif, 684LTVP\(^6\)\(^8\)\(^7\) (human) ~30 amino acids N-terminal to the PxxIxIT motif (Fig. 1c). Further, the alignment showed that the LxVP motif is considerably more conserved than the PxxIxIT motif, supporting its potential importance in CN binding and NHE1 function.

To test if the putative LxVP motif in NHE1 (684LTVP\(^6\)\(^8\)\(^7\)) binds directly to CN, we used NMR spectroscopy. An overlay of the 2D [\(^1\)\(^H\),\(^1\)\(^5\)\(^N\)] HSQC spectrum of \(^1\)\(^5\)\(^N\)-labeled NHE1ct in the presence and absence of CN showed that multiple peaks disappeared upon complex formation (Fig. 2a). Specifically, cross-peaks corresponding to NHE1 residues 684LTVP\(^6\)\(^8\)\(^7\) (684, 688, 686) and 715PVITID\(^7\)\(^2\)\(^0\) (717, 719) were significantly broadened partially beyond detection upon binding CN. In addition, many of the peaks originating from the 27 residues that connect the NHE1 LxVP and PxxIxIT motifs were also broadened upon complex formation (Fig. 2a, b), indicating either that this region is involved in the interaction with CN or that the conformational freedom of the linker is impaired by the anchoring of both SLiMs to CN\(^2\)\(^4\)\(^2\)\(^5\). Thus, NHE1\(^6\)\(^8\)\(^4\)\(^T\)PVTIP\(^6\)\(^8\)\(^7\) is a CN-specific LxVP SLiM that, together with NHE1 715PVITID\(^7\)\(^2\)\(^0\), binds to CN.

To determine the contribution of each SLiM to CN binding, we used isothermal titration calorimetry (ITC; Table 1). CN and NHE1ct formed a tight 1:1 complex (\(K_D = 59 ± 9\) nM; Fig. 2c).

Mutating either the NHE1 LxVP (NHE1ct\(_{ATAP}\)) or PxxIxIT (NHE1ct\(_{AVATAA}\)) motifs resulted in a significant reduction in binding affinity (Fig. 2d, e). Specifically, NHE1ct\(_{ATAP}\) bound CN with a \(K_D\) of 4.8 ± 0.5 \(\mu\)M (~80-fold reduction vs. WT), while NHE1ct\(_{AVATAA}\) bound CN with a \(K_D\) of 2.4 ± 0.5 \(\mu\)M (~40-fold reduction vs. WT). Similarly, short NHE1 LxVP and PxxIxIT peptides bound CN with \(K_D\) values of 4.3 ± 0.5 and 6.5 ± 0.5 \(\mu\)M, respectively (Fig. 2f, g). The increase in the \(K_D\) for the NHE1 peptides versus the corresponding binding compromised variant (NHE1ct\(_{ATAP}\) includes only a functional PxxIxIT site; NHE1ct\(_{AVATAA}\) includes only a functional LxVP site) suggests a contribution by the linker and/or distal residues to the overall binding affinity. Finally, the large increase in affinity observed for NHE1ct compared to the affinities of each peptide or NHE1ct motif variants demonstrates avidity.

**NHE1:CN interaction in cells.** To determine if both the LxVP and PxxIxIT motifs are important for NHE1:CN binding in cells, we stably expressed either full-length NHE1\(_{WT}\), NHE1ct\(_{ATAP}\), or NHE1ct\(_{AVATAA}\).
NHE1<sub>AVATAA</sub> or NHE1<sub>AVATAAATAP</sub> in PS120 mammalian fibroblasts (Fig. 2h, Supplementary Fig. 2; these cells lack endogenous NHE1). As expected from in situ proximity ligation assays (PLA), which confirmed the close proximity (<40 nm) of endogenous NHE1 with endogenous CN in MCF-7 breast cancer cells (Fig. 2i, j), stably expressed WT NHE1 robustly co-immunoprecipitated with CN in PS120 cells, with and without an increase in cellular [Ca<sup>2+</sup>], (Fig. 2h, Supplementary Fig. 2). Similar amounts of NHE1 co-immunoprecipitated with CN for the variants NHE1<sub>ATAP</sub> and NHE1<sub>AVATAA</sub>, whereas NHE1<sub>AVATAAATAP</sub> co-immunoprecipitated less with CN than WT NHE1. These data together with our NMR and ITC data (Fig. 2a–g) demonstrate that both the NHE1 LxVP and PxIxIT motifs contribute to CN binding.

Sequence differences in CN-binding motifs fine-tune CN binding. To understand how NHE1 binds CN at a molecular level, we used ITC and X-ray crystallography (Fig. 3a, b). First, we used ITC to show that NHE1 residues I680–S723 constitute the minimal NHE1 CN-binding domain (NHE1 residues I680–S723, hereafter referred to as NHE1<sub>ct</sub>Δ). Then, we determined the three-dimensional structure of the NHE1<sub>ct</sub>Δ-CN complex to a resolution of 1.9 Å (CN:−370, CNB<sub>16:−170</sub>; Supplementary Table 1). In addition to the electron density observed for CN, strong electron density was observed for NHE1 residues N681–A688 (LxVP motif) and D713–S723 (PxIxIT motif) (Fig. 3a). The NHE1 LxVP and PxIxIT motifs bind CN in the canonical LxVP and PxIxIT-binding pockets, both of which are ~35 Å distal to the CN active site.

The interaction of NHE1 with the CN PxIxIT-binding pocket, which buries 626 Å<sup>2</sup> of solvent accessible surface area (SASA), is similar to that observed in other CN–PxIxIT complexes (Fig. 3c). Namely, the NHE1 PxIxIT motif forms a β-strand that hydrogen bonds with β14 of CNA, extending one of its central β-sheets. Typically, the PxIxIT interaction is further stabilized by multiple hydrophobic contacts between the two conserved I residues. A comparison of the NHE1–PxIxIT interaction with CN to other CN–PxIxIT complexes revealed that the hydrophobic interaction is unique and non-optimal (Fig. 3d). Specifically, in the NHE1-CN complex, both NHE1 PxIxIT residues T718 and D720 form hydrogen bonds with CNA N330. This alters the NHE1 backbone conformation (likely facilitated by the restricted Φ/Ψ space due to NHE1 P721), causing the sidechain of I719 to project out of the hydrophobic CNA PxIxIT-binding pocket (CNA M290, Y288, I331). To confirm this, we mutated the NHE1 PxIxIT motif to a canonical, optimal sequence (PVIVIT), which bound CN five-fold more tightly than WT NHE1<sub>ct</sub>Δ (Supplementary Fig. 3A; Table 1). This non-optimal hydrophobic interaction of NHE1 1719 with CN explains the unusually weak binding of the NHE1 PxIxIT motif to CN, as measured using ITC and co-IP experiments.

The interaction of NHE1 with CN at the LxVP-binding pocket, which buries 560 Å<sup>2</sup> of SASA, is essentially identical to those observed between CN and other LxVP motifs<sup>21</sup>. The interaction is dominated by NHE1 LxVP residues L684 and V686, which bind CN in two deep hydrophobic pockets formed at the CNA/B interface (Fig. 3e). Interestingly, and different to the interaction observed between CN and the NFATC1-LxVP<sub>peptide</sub> peptide<sup>26</sup>, residues N-terminal to the NHE1 LxVP motif also contribute to binding, albeit in a slightly different manner. Specifically, NHE1 N682 (--2 relative to LxVP) forms a weak hydrogen-bond with CNB Q50, while NHE1 N681 (--3) forms a hydrogen-bond with CNA K360. This shows that residues flanking LxVP motifs also contribute to CN binding.
The motif-connecting linker dynamically interacts with CN. Despite the observation that most of the NHE1 residues linking the LxVP and PxIxIT motifs exhibited line broadening (Fig. 2a, b), no electron density was detected for residues H689-E712 and their corresponding peptides that report increases in affinity for CN using ITC. While the interaction affinity is unchanged (Supplementary Fig. 3B; Table 1), a change in the distance between the C-terminal residue of the LxVP motif and the N-terminal residue of the PxIxIT motif is too short to allow the linker to span the back side of CN, the structure also reveals that the dynamic linker connects the motifs by spanning the front, active-site containing side of CN.

Modeling potential paths that the linker may take to connect the two motif binding pockets revealed a set of basic residues in NHE1 (I680-R708) ideally positioned to electrostatically interact with multiple acid patches (AP) on CN (AP1: E205/D211/D229; AP2: E246; AP3: E237/D238; Fig. 3f). To determine if NHE1 residues 698-RAR700 contribute to CN binding, we generated the variant NHE1ctA392R (NHE1ctA392R, which lacks the ability to engage in charge:charge protein:protein interactions, and then measured its affinity for CN using ITC. While the affinity is unchanged (Supplementary Fig. 3B; Table 1), a change
in TΔS indicated reduced flexibility in the charge neutral interaction; thus, NHE1 R698 and R700 likely mediate dynamically important electrostatic interactions with CN.

To confirm these results, we used NMR spectroscopy. An overlay of the 2D [1H,15N] HSQC spectrum of 15N-labeled NHE1ct92RARARARA in the presence and absence of CN showed that nearly all peaks broadened beyond detection (Supplementary Fig. 4). This confirms the ITC results of reduced flexibility. We also determined the 3D structure of the NHE1ct92RARARARA-CN complex to a resolution of 1.9 Å (Supplementary Fig. 5; Supplementary Table 1). Electron density is observed for CN and for NHE1 residues I681–A688 (LxVP motif) and D713–S723 (PxIxIT motif) but not for the linker; this shows that the intervening linker does not form a single conformation that can be identified using X-ray crystallography and highlights the importance of using complementary biophysical techniques for studying dynamic interactions.

CN specifically dephosphorylates NHE1 pT779. Having established that NHE1 binds CN via both a PxIxIT and LxVP motif, we asked whether NHE1 is simply a CN scaffold or is additionally a CN substrate. Recently, we used NMR-based phosphorylation assays to show that the NHE1ct is phosphorylated by ERK2 at multiple sites (pS693, pS723, pS726, pS771, pT779, pS785, Fig. 4a). These sites are phosphorylated similarly by other MAPKs (p38α and JNK1; Supplementary Fig. 6). Here, we used NMR spectroscopy to monitor CN-mediated dephosphorylation...
of phosphorylated NHE1ct. Of these six ERK2 phosphorylated residues, only pT779 was specifically and rapidly dephosphorylated by CN (Fig. 4a, b; Table 2, Supplementary Table 2).

To explore if phosphorylation of T779 impacts NHE1 function, WT NHE1 and the variants NHE1 T779A (prevents phosphorylation) and NHE1 T779D (mimics phosphorylation) were expressed in PS120 cells that lack endogenous NHE activity. All NHE1 variants localized to the plasma membrane similarly to WT NHE1 (Fig. 4c, Supplementary Fig. 7) and expressed to similar levels (Fig. 4d, e). One-way ANOVA with Dunnet’s post-test (n = 5 biologically independent experiments for all cell lines except 779, where n = 3). * and *** denotes p < 0.05 and p < 0.001, respectively. Error bars represent S.E.M. values. Source data are provided as a Source Data file.
Table 2 Apparent in vitro dephosphorylation rates of NHE1 by CN

| NHE1ct or CN variant | Site | Phosphosite sequence$^a$ | $k_{\text{dephos}}$ \((10^{-3} \text{ h}^{-1})^{-1}\) |
|----------------------|------|--------------------------|------------------|
| NHE1ct WT and variants$^b$ | pT779 | **777SGTDVFTPAPS**[PS5787]** | **98 ± 3** |
| NHE1ct | pT779 | **777SGTDVFTPAPS**[PS5787]** | **11 ± 3** |
| NHE1ct motif variants | pT779 | **777SGTDVFTPAPS**[PS5787]** | **642 ± 28** |
| NHE1ctAVATAA | pT779 | **777SGTDVFTPAPS**[PS5787]** | **811 ± 55** |
| NHE1ctATAP | pT779 | **777SGTDVFTPAPS**[PS5787]** | **199 ± 10** |
| Role of Thr vs. Ser in CN-mediated dephosphorylation | pT779S | **777SGTDVFSPAPS**[PS5787]** | **1 ± 2** |
| NHE1ct | pT779 | **777SGTDVFTPAPS**[TP5787]** | **193 ± 5** |
| NHE1ct | pT779 | **777VFTAPS**[P5787]**PSQRIQ792** | **35 ± 7** |
| Role of TxxP motif in CN-mediated dephosphorylation | pT779S | **777SGTDVFPAPS**[PS5787]** | **28 ± 3** |
| NHE1ct | pT779 | **777SGTDVFTAPS**[TPA5787]** | **102 ± 7** |
| NHE1ct | pT779 | **777VFTAPS**[PAPA5787]**PSQRIQ792** | **96 ± 7** |
| CN variants$^c$ | pT779 | **777SGTDVFTPAPS**[PS5787]** | **347 ± 10** |
| CNA | pT779 | **777SGTDVFTAPS**[PS5787]** | **60 ± 10** |

$^a$Sequence: dephosphorylated residue (underlined)

$^b$All reactions performed using NHE1ct and the CN constructs indicated

$^c$All reactions performed using CN and the NHE1ct constructs indicated

$^d$All reactions performed using CN and the NHE1ct constructs indicated

$^e$Apparent rates of dephosphorylation were extracted from global non-linear least-square fits of disappearing peaks (dephosphorylation) to single exponentials in SigmaPlot. The standard errors ($\pm$) of the estimated parameters were reported to represent confidence intervals.

absence of HCO$_3^−$. Under these conditions, recovery from acidification represents the activity of the exogenously expressed NHE1. As a control, untransfected PS120 cells showed no pHi recovery (Fig. 4f, g). Cells expressing the NHE1 T779D phosphomimetic mutant had a pHi recovery rate that was ≥3-times faster than cells expressing WT NHE1. By comparison, NHE1 T779A showed a non-significant increase in pHi recovery rate. Furthermore, cells expressing the NHE1 ATAP/AVATAA mutant also exhibited increased pHi recovery rate compared to WT NHE1. Together, these results show that the interaction with CN, and specifically the dephosphorylation of NHE1 pT779, negatively regulates NHE1 activity, and conversely, that phosphorylation of T779 increases the transport activity of NHE1.

Specificity of CN for pT779 is not due to Thr preference. Having established that NHE1 T779 is a functionally important phosphorylation site and that its regulation by CN is essential for NHE1 function in cells, we set out to identify the molecular mechanisms by which CN specifically recognizes and dephosphorylates NHE1 pT779. The inability of CN to dephosphorylate pS693, pS726, and pS723 is explained by their close proximity to the NHE1 LxVP and PxIXIT motifs (Figs. 1c and 3a), which sterically prevents them from reaching the CN active site. However, both NHE1 pS771 and pS785, along with pT779, are ≥50 residues C-terminal to the NHE1 PxIXIT motif. Because all three residues can readily reach the CN active site, additional mechanisms must be responsible for restricting CN activity to only NHE1 pT779.

PPPs preferentially dephosphorylate Thr over Ser, although the underlying molecular mechanism is unknown. Thus, one possibility for the preferred dephosphorylation of pT779 is that it is a Thr (Fig. 5a). To test this, we repeated the dephosphorylation experiments using a NHE1ct T779S variant. As expected, CN dephosphorylated pT779S more slowly than pT779 (WT) (Fig. 5b). However, the dephosphorylation of pT779S was still faster than the rate measured for any other NHE1ct phosphorylated Ser residues (pS693, pS723, pS726, pS771, pS785; Fig. 4b; Table 2, Supplementary Table 2). To further confirm the specificity of CN for Thr, we also generated a NHE1ct S785T variant. CN did dephosphorylate pS785T; however, the rate of dephosphorylation was much slower than that observed for pT779 (Fig. 5c; Table 2, Supplementary Table 2). Thus, while these data confirm the preference of CN for Thr over Ser residues, generally, the enhanced dephosphorylation of pT779 specifically is not solely explained by this preference.

The motifs differentially modulate NHE1 dephosphorylation. To determine the role(s) of the PxxIIT and LxVP motifs for dephosphorylation of NHE1ct, we repeated the NMR time course experiments using the NHE1ct ATAP, NHE1ct AVATAA, and NHE1ct ATAP AVATAA variants (Fig. 5d; Table 2, Supplementary Table 2). The data showed the following. First, abolishing the NHE1 PxxIIT-CN interaction (NHE1ct AVATAA) reduced the dephosphorylation rate of pT779 (no additional sites were dephosphorylated). Second, abolishing the NHE1 LxVP-CN interaction (NHE1ct ATAP) increased the dephosphorylation rate of pT779, accompanied by slow dephosphorylation of pS771 and pS785 (Table 2, Supplementary Table 2). Third, dephosphorylation of NHE1ct ATAP AVATAA resembled that of NHE1ct ATAP with additional slow dephosphorylation of pS693, pS723, and pS726 (Table 2, Supplementary Table 2). Fourth, to test the contribution of the LxVP interaction, we repeated the dephosphorylation of pT779 using only CNA, which lacks the LxVP-binding pocket (this pocket is only present when CNB is bound to CNA via CNA residues 348–370). Again, the dephosphorylation rate of pT779 was significantly faster (Fig. 5e; Table 2, Supplementary Table 2). Finally, we tested the contribution of electrostatics and thereby the dynamics of the 27-residue linker using NHE1ct BARRA (NHE1ct 698AAA700), which led to a 1.5-fold increased rate of pT779 dephosphorylation (Supplementary Fig. 8A; Table 2). This shows that the 27-residue linker directly negatively influences the rate of pT779 dephosphorylation.

These dephosphorylation results are fully consistent with our molecular data. First, loss of binding at the PxxIIT site (CN with
NHE1ctAVATAA lead to an overall reduction in the dephosphorylation rate of pT779 without loss of specificity, most easily explained by the increased distance between the substrate site (pT779) and the closest docking site on CN for this variant, i.e. the LxVP motif. In contrast, loss of binding at the LxVP site (CN with NHE1ctATAP or NHE1ctATAP/AVATAA; NHE1ct with CNA) negated the unique specificity for pT779. This effect most likely stems from an abolished interaction between CN and the 27-residue linker that connects the NHE1 LxVP and PxIxIT-binding motifs. As established, this linker interacts in a dynamic manner with CN. To confirm this model, we repeated the dephosphorylation of NHE1ctATAP by CN in the presence of a large excess of LxVP peptide (CN:NHE1ctATAP: LxVPpeptide molar ratio of 1:100:100). Based on the respective concentrations and affinities, this should result in nearly 100% occupancies of both the LxVP and PxIxIT-binding sites (by the LxVPpeptide and NHE1ctATAP, respectively). Again, a much faster dephosphorylation rate was observed (Fig. 5f; Table 2), further supporting that the 27-residue linker connecting the LxVP and PxIxIT sites restricts access to the CN active site and that anchoring the linker via the LxVP CN pocket controls this specificity.

Our dephosphorylation data show that the presence of the linker not only reduces the dephosphorylation rate of pT779 but also increases the specificity of CN for only pT779. This is because slow dephosphorylation of pS771 and pS785 is only observed in the absence of linker-anchoring at the LxVP site (i.e. with NHE1ctATAP). Considering the difference in co-localization, these results are consistent with an observed increase in acidification recovery in NHE1 motif variants compared to WT NHE1 when expressed in PS120 cells (Fig. 4f, g).

A TxxP motif is a preferred substrate for CN. While the NHE1 linker influences CN dephosphorylation specificity and rates, an activity that requires the LxVP interaction, these interactions are
The balance between phosphorylation and dephosphorylation

**Molecular basis for TxxP recognition.** Next, we created a model where we replaced the CN AID 481ERMP1484 sequence with the NHE1 779TPAP782 sequence (Fig. 5i). We speculated that the affinity of the NHE1ct P782A variant for CN was unchanged (Supplementary Fig. 3C; Table 1). In spite of the identical affinity, the dephosphorylation of pT779 was impaired in this variant (Fig. 5g; Table 2), demonstrating the proline in the i+3 position (P782) is essential for robust dephosphorylation. To confirm the role of the TxxP motif as a CN-specific substrate determinant, an additional TxxP motif was introduced at a different NHE1ct site. Specifically, the NHE1 S785 sequence was replaced with a TxxP sequence (784DPSSQR790) to DTPAPQDB and the dephosphorylation analysis repeated (Fig. 5b). The dephosphorylation rates for the Thr residues in both TPAP motifs (T779 and S785T, both phosphorylated residues) are similar (Table 2, Supplementary Table 2). This strongly supports the role of the motif as a bona fide substrate determinant for CN.

**Discussion**

The balance between phosphorylation and dephosphorylation controls cell signaling and cellular communication. Ser/Thr kinases, the enzymes catalyzing phosphorylation, use protein:protein interactions and substrate-specific recognition sequences to identify their substrates. Conversely, the mode(s) of substrate recognition for the PPP Ser/Thr protein phosphatase family (PP1, PP2A, PP2B/3, PP4, PP5, PP6, and PP7) is not known. While it has become clear that at least PP1, PP2A, PP2B, and PP4 utilize protein:protein interactions for substrate binding, it is still unclear if and how these interactions direct substrate specificity for single dephosphorylation sites, often 50 residues or more away from the scaffolding motifs. Our lack of understanding of molecular selectivity determinants in PPPs hampers bioinformatics analysis to assign PPP:substrate pairs, thus impairing progress in obtaining a systems biology understanding of signaling.

One protein that is tightly regulated by both protein:protein interactions and post-translational modifications is NHE1, an evolutionarily conserved acid-extruding transporter serving as a key regulator of cellular pH~i in essentially all mammalian cells studied. Six NHE1ct residues are phosphorylated by ERK2, a kinase that is often activated downstream of, or in parallel with, increases in Ca2+. A previous report showed that an NHE1 T779A/S785A mutant exhibited a tendency for reduced NHE1 activation by sustained acidosis. We show here that dephosphorylation of T779 increases NHE1 activity and, in turn, pT779 is dephosphorylated by the Ca2+-activated phosphatase, CN. This suggests the existence of a feedback mechanism, in which the extent and duration of NHE1 activation by ERK are restricted by its dephosphorylation following [Ca2+]i-induced CN activation. As excessive NHE1 activation is associated with, e.g. uncontrolled cell division and growth, this intrinsic brake on its activity is likely physiologically meaningful. In conjunction with previous work showing that CN activity in vivo is regulated by NHE1-dependent pH~i changes, this reveals the existence of a dynamic complex of mutual regulatory interactions with the NHE1–CN interaction at its core.

We show that the two canonical CN-specific SLIMs, a previously identified PtxIxIT motif and a newly discovered LxVP motif, contribute to NHE1 binding of CN. As observed for other CN regulators and inhibitors, minor changes in these motifs lead to significant changes in interaction strength. Interestingly, mutation of both sites was insufficient to abolish endogenous CN binding to full-length NHE1 in a cellular context—likely reflecting the known, extensive scaffolding properties of NHE1, which forms dynamic, multiprotein complexes at the plasma membrane. Further, in NHE1, the LxVP sequence (884TVP887) is not only an LxVP motif but also functions as a D-domain/Kinase Interaction Motif (KIM) that is essential for ERK2 binding and scaffolding. The use of an overlapping sequence ensures that ERK2 and CN binding to NHE1 is mutually exclusive.

We also discovered that the LxVP motif is essential for directing the specificity of CN for NHE1 pT779. However, it does so in an unanticipated and indirect manner. Namely, NHE1 forms a critical anchor for a 27-residue linker that dynamically associates with CN across its front surface. In doing so, it reduces access to the CN active site, which in turn, reduces the overall speed of dephosphorylation. Simultaneously, this increases the specificity towards a single phosphorylated residue, pT779. While it has been known for more than 30 years that CN prefers its Thr residues of Thr, TxxP) makes a specific and unique interaction with a CN surface binding pocket formed by CN residues H155 and Y159. These residues are not conserved in any other PPP, i.e., the specific dephosphorylation of the TxxP motif is unique to CN. Taken together this shows that CN uses a combination of mechanisms, including a dynamic interaction...
with a 27-residue linker that restricts substrate access to a unique interaction pocket adjacent to the catalytic site leading to a preference for TxxP motifs, to achieve exquisite specificity towards a single Thr in NHE1, resulting in rapid and specific dephosphorylation of this residue, hence limiting the duration of NHE1 activation following its ERK-induced phosphorylation. This is furthermore a remarkable example on how disordered linkers in complexes have direct functional relevance, in this case acting as a single Thr in NHE1, resulting in rapid and specific for TxxP motifs, to achieve exquisite specificity.

For protein expression, NHE1ct and CN were expressed as described [21-2]. Briefly, proteins were expressed in E. coli BL21 (DE3) cells (Agilent). Cell cultures were grown at 37 °C under vigorous shaking (250 rpm) to an OD600 of 0.7. Cells were cooled at 4 °C for one hour, while the shaker temperature was lowered to 18 °C. Expression was induced by addition of 1 mM IPTG, and the cultures were grown for an additional 18 h at 18 °C (250 rpm). The cells were harvested by centrifugation (60000 × g, 15 min, 4 °C) and stored at −80 °C. NHE1ct was grown in E. coli BL21 (DE3) CodonPlus-RIL cells (Agilent). Cells were grown in Luria Broth in the presence of selective antibiotics at 37 °C up to OD600 of 0.6–0.8, and expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). NHE1ct was expressed for 4 h at 37 °C and CN was expressed for 18 h at 18 °C prior to harvesting by centrifugation at 6000 × g. Cell pellets were stored at −80 °C for further purification. For NMR measurements, expression of uniformly 13N-labeled and/or 15N-labeled NHE1 was achieved by growing cells in M9 minimal media containing 1g/l. [1³N]-Cl and/or 4g/l [1⁵N]-D-glucose (CL or Iotoc) as the sole nitrogen and carbon sources, respectively.

CN and NHE1ct were purified as described [21-2]. For all protein purification pellets were lysed in lysis buffer (20 mM Tris–HCl pH 8.0, 500 mM NaCl, 5 mM Imidazole, 0.1% Triton X-100) containing EDTA-free protease inhibitor cocktail (Roche) using high-pressure homogenization (Avetin). The lystate was clarified at 45,000 × g for 60 min at 4 °C. The supernatant was filtered through a 0.22 μm filter before loading onto a 5 ml HiTrap HP column (GE Healthcare) equilibrated in Buffer A (50 mM Tris–HCl pH 8.0, 500 mM NaCl, 5 mM imidazole, 0% D2O). Protein bands were washed with Buffer A and were eluted using a linear gradient of Buffer B (50 mM Tris–HCl pH 8.0, 500 mM NaCl, 0.5 mM imidazole) with 0.5 mM TCEP with TCEP to cleave the His6-tag. The next day, the protein was incubated with Ni²⁺-NTA resin (GE Healthcare) to remove TEV and the cleaved His-tag. Cleaved NHE1ct or NHE1ctΔ352 was incubated at 80 °C and centrifuged at 14,000 × g for 10 min, the supernatant was purified using size-exclusion chromatography (SEC, Superdex 75 26/60) with 1 mM EDTA to SEC (Superdex 75 26/60, GE Healthcare), equilibrated in SEC buffer; 20 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM CaCl₂, and 0.5 mM TCEP) and concentrated to 1–2 mg/ml prior to storage at −80 °C. CN was purified identically, except the cleaved product was further purified by anion exchange chromatography (HiTrap QHP, salt loading buffer: 20 mM Tris–HCl pH 7.5, 50 mM NaCl, 1 mM CaCl₂, and 0.5 mM TCEP, high salt elution buffer: 20 mM Tris–HCl pH 7.5, 800 mM NaCl, 1 mM CaCl₂, and 0.5 mM TCEP) to SEC (Superdex 75 26/60, GE Healthcare), equilibrated in SEC buffer; 20 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM CaCl₂, and 0.5 mM TCEP).

To form NHE1ct:CN complexes, CN (CNA-1370, CNB-16170) was purified as described above except fractions containing CN after the anion exchange chromatography step were combined and incubated with a three-fold molar excess of NHE1ctΔ352 or NHE1ctΔ352Δ92PVIVIT,C (alpha isoform, subunit A: M1-A391, H155A, Y159A, subunit B: 1–170, CNA/CNB (alpha isoform, subunit A: M1-N370, H155A, Y159A, subunit B: 16–170), CNA (alpha isoform: H27-D348: two C-terminal ASP residues were added for increased CNA solubility).

**Protein expression and purification.** DNA coding the human NHE1 1680–Q815 (NHE1ct) was subcloned into a PET-11a expression vector [21]. DNA coding the rat NHE1Δ352 was subcloned into a PET-33b expression vector. Both vectors include an N-terminal His-tag follow by maltose-binding protein (MBP) and a TEV (tobacco etch virus) protease cleavage site. The previous human NHE1 (see also Supplementary Fig. 1) and human CN variants were generated using the Quick-change (Agilent) site-directed mutagenesis step. NHE1ctΔ352, LxVP, CNΔ92PVIVIT,C was relative to 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) and spectra were referenced at 5 °C (interaction) or 25 °C (time-course experiments). Chemical shift referencing (Bruker BioSpin) and the spectra analyzed using both the CCPN Analysis software [35] and CARA [36] (http://www.cara.nmr.ch). Interaction and time course NMR data were acquired at 25 °C on a Varian INOVA 800 MHz (H) spectrometer with a room temperature probe or a Bruker AVANCE/ NEO 600 or 750 MHz (H) spectrometer equipped with cryogenic probes. Free induction decays were transformed and visualized in NMRPipe [34] or Topspin 4.05 (Bruker BioSpin) and the spectra analyzed using both the CCPN Analysis software and CARA [36] (http://www.cara.nmr.ch). Interaction and time course experiments were recorded on Varian 800 MHz (H) NMR spectrometers with a 5 mm triple resonance probe with a Z-field gradient or a Bruker NEO 600 MHz (H) NMR spectrometer with a 5 mm TCI-active HCX z-gradient cryoprobe at 5 °C (interaction) or 25 °C (time-course experiments). Chemical shift referencing was performed with d4-1,4-dimethoxybenzene as the internal standard. Spectra were zero filled, Fourier transformed and baseline corrected in NMRDraw [34] or Topspin 4.05 (Bruker BioSpin) and analyzed in CCPN Analysis [35]. For the phosphorylation experiments using different MAPKs, NMR samples of 400 μL of 200 mM 13N-labeled NHE1ct were prepared in PBS buffer pH 7.0, 5 mM ATP, 10 mM MgCl₂, 0.1% CHAPS, 50 mM HCl pH 8.0, 500 μM DTT, 10% (v/v) 99.96% D2O. [1³N]-HSCQ spectra were recorded before and after incubation with the active kinase (purchased from proteinkinase.de) for 48 h at 25 °C (1 µg of active ERK2, 20 µg of active JNK1).

**Methods**

**Sequence alignments.** ClustalW was used to create all sequence alignments. The following species were used in Fig. 1: *Amphitoxus trifasciatus* (atNHE1; salmon); *Cricetulus griseus* (cgNHE1; Chinese hamster); *Sus scrofa* (ssNHE1; pig); *Bos taurus* (btNHE1; cow); *Homo sapiens* (hNHE1; human), *Oryctolagus cuniculus* (onNHE1; rabbit), *Gallus gallus* (ggNHE1; chicken), *Takifugu rubripes* (tnNHE1; Japanese pufferfish), *Pleuronectes americanus* (paNHE1; winter flounder), *Platichthys flesus* (pNHE1; European flounder); *Onchorhynchus mykiss* (omNHE1; rainbow trout), and *Cyprinus carpio* (ccNHE1; carp).

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were taken as z-stacks and z-projection images were created. Further image processing included filtering, equalization, and unsharp masking. Confocal images were deconvolved using the autoXDS42,43, or SAINT/XPREP (Bruker AXS Inc., Madison, 2004). All three algorithms were used and the best data set was selected. The final images were processed using the ColorProfiler Image software plugin.

Proximity ligation assays. Proximity ligation assay was carried out using a Duolink Detection Reagents Red kit (Sigma Aldrich). WT MCF7 cells were seeded on coverslips the day before assay (MCF7-7 cells were a kind gift from Dr. Jacques Pouyssegur [University of Nice, Sophia Antipolis, France]). Prior to IP, cells were subjected to a 10 min incubation with cold phosphate buffered saline, permeabilized with DAPI (using only anti-CN primary antibody; NHE1: XB-17, 1:100 (gift from Mark Musch, University of Chicago); CN: Millipore-Sigma, #C1956, 1:200) and NHE1-truncating mutations were visualized using the ×60 s NA objective of an Olympus BX fluorescence microscope using cellSens Dimensional V1.6 software. Images were deconvolved using the autoXDS42,43, or SAINT/XPREP (Bruker AXS Inc., Madison, 2004). All three algorithms were used and the best data set was selected. The final images were processed using the ColorProfiler Image software plugin.

Crystallization, data collection, and structure determination. The NHE1ct92:CN complex (and variants) was concentrated to 8–10 mg/ml in 10 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM CaCl₂, and 0.5 mM TCEP. Initial crystallization trials were carried out using sitting drop vapor diffusion yielded needle-like crystals in 40% (v/v) PEG 600, 100 mM CHES/sodium hydroxide pH 9.5 at RT. Optimization was performed using the initial crystal hits as seed stock for seeding. The final crystals were obtained from seeding in 40% (v/v) PEG 600, 100 mM CHES/sodium hydroxide pH 9.5, 0.2 M MgCl₂. Crystals were cryoprotected using a 15 s-soak in mother liquor and immediately flash frozen in liquid nitrogen. Data were collected at SSRL (beamline 12-2; NHE1ct92:CN) or the University of Arizona (Bruker liquid Gallium MetalJet X-ray Diffractometer with a Photon II CPAD detector; NHE1ΔC/CN and NHE1ct92:CNΔL) and analyzed using NITPIC and Solve (Bruker AXS, 2004). All three algorithms were used and the best data set was selected. The final images were processed using the ColorProfiler Image software plugin.

Co-Immunoprecipitation. Co-immunoprecipitation (IP) was carried out for fully confluent PS120 cells, expressing WT47, variant NHE1 or empty vector (PS120 cells were generated in the laboratory of Dr. Jacques Pouyssegur [University of Nice, France]) and were a kind gift from Prof. Laurent Counillon [University of Nice Sophia Antipolis, France]). Prior to IP, cells were subjected to a 10 min incubation in physiological saline (Ringer solution, 135 mM NaCl) in the absence or presence of 5 µM CN (Sigma-Aldrich) or 0.1% DAPI. Cells were washed with PBS and lysed in pre-heated IGEPAL lysis buffer (1% (v/v) IGEPAL CA-630, 1 mM NaF, 3 mM NaN₃, EDTA-free Complete™ protease inhibitor, 50 mM Tris pH 7.4, and 140 mM NaCl), detached using a rubber policeman and incubated on ice for 30 min. Preparation of lysates and determination of protein contents was done as described for immunoblotting. 2 mg protein for each IP were incubated with primary antibodies against CN and NHE1, or IgG control (1 µg antibody per mg lysate) for 30 min at 4 °C. Lysates were then washed with DAPI and incubated for 30 min at 4 °C with gentle rotation. Beads were pelleted by magnetic separation and washed with bound protein was washed 5 x 2 min in 500 µl lysis buffer, boiled for 5 min at 95 °C with 80 µl sample buffer, mixed thoroughly, and incubated on ice for 30 min to ensure full elution. Proteins were separated using SDS–PAGE and analyzed by Western blotting as above. Input samples were analyzed in parallel.

Immunoblotting. Cells were grown to ~80% confluence in 10 cm Petri dishes, washed in ice-cold PBS, lysed in boiling lysis buffer (1% SDS, 10 mM Tris–HCl pH 7.5), sonicated, and centrifuged to clear debris. Identical amounts of protein (15–25 µg/well) diluted in NuPAGE LDS sample buffer (LifeTech Technologies) were boiled for 5 min, separated on NuPAGE 10% bis-tris gels, and transferred to nitrocellulose membranes using a Novex gel transfer system. Membranes were stained with Ponceau S to confirm equal loading, blocked for 1 h at 37 °C in blocking buffer (120 mM NaCl, 10 mM Tris–HCl, 5% nonfat dry milk), and incubated with the relevant primary antibodies in blocking buffer overnight at 4 °C. After washing in TBST (0.1% Tween-20), membranes were incubated with HRP-conjugated secondary antibodies (1:2500, Sigma), washed in TBST, and visualized using BCIP/NBT. For quantitative analyses, blots were scanned and bands intensities quantified using Un-Scan-IT Graph Digitizer software (Silk Scientific). Protein bands were normalized to those of the loading control (p150) from the same gel to eliminate gel-to-gel differences. For immunoprecipitation samples, protein bands were normalized to the input, from the same gel. Uncropped and unprocessed scans of the blots are provided in the Source Data file. Primers and recombinant DNA used in this study are summarized in Supplementary Table 4.

Immunofluorescence analysis of NHE1 and CN. For immunofluorescence experiments, cells were grown on 12 mm round glass coverslips until ~80% confluence and fixed in 2% MeOH (15 min on ice). Coverslips were washed three times with PBS, permeabilized with 0.5% Triton-X 100 in TBS, and blocked using O-link-blocking solution for 60 min at 4 °C. Covers were washed in Duolink II Buffer A and incubated with ligase diluted 1:40 in 1x Duolink ligation solution for 30 min at 4 °C, followed by incubation with DAPI probes diluted 1:5 in Duolink II Antibody Diluent buffer at 37 °C for 30 min. Coverslips were washed in Duolink II Buffer A and incubated with ligase diluted 1:40 in 1x Duolink ligation solution for 30 min at 37 °C, followed by wash in Duolink II Buffer A. Subsequently, coverslips were incubated with a polymerase diluted 1:80 in 1x Duolink amplification solution for 100 min at 37 °C. After amplification, coverslips were washed in Duolink Buffer A, incubated with phallolidin-FluoSpheres 1 µg/ml (1 h, at RT) and labeled with DAPI. Finally, coverslips were washed in Duolink Buffer A and MQ, mounted on object glass with mounting medium, and sealed with nail polish. Imaging was carried out with an Olympus BX-61 epifluorescence microscope using cellsDimensions V1.6 software. Images were deconvolved using the autoXDS42,43, or SAINT/XPREP (Bruker AXS Inc., Madison, 2004). All three algorithms were used and the best data set was selected. The final images were processed using the ColorProfiler Image software plugin.
length of 0.3 cm (96-well plates). $K_m$ and $V_{max}$ were determined by fitting to the Michaelis–Menten equation, $y = V_{max}x/(K_m + x)$; $k_{cat}$ was extracted using $y = E_0^x/(E_0 - x)$ (Sigma Plot 13). The catalytic efficiency was obtained as $k_{cat}/K_m$. All experiments were carried out in triplicate.

**Bioinformatics.** The CNcon database was used for screening for the TxxP in other CN substrates. ScanProsite was used to identify LxVP motifs for proteins in the CNcon database with an LxVP site using [S/T]xxP filters for IDR behavior using different IDP predictors including IUPred. To identify the distance between C-terminal LxVP and [S/T]xxP sites, a minimum cut-off of 9 residues was used, as this is sufficient to span the distance between the CN LxVP-binding pocket and the CN active site.

**Measurements of pH.** Measurements of pH were performed using the ammiamo pre-pulse technique. Briefly, 7×10^4–10^5 PS120 WT-expressing or variant NHE1-expressing cells were seeded in 24-well plates 24 h prior to the experiment, then loaded with 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetox-

**Cell surface biotinylation.** To determine the surface fraction of all NHE1 variants, cells were incubated for 30 min at 4 °C with freshly made EZ-Link Sulfo-NHS-SS-Biotin (Life Technologies, #21331) diluted in PBS, followed by washing in cold quenching buffer (0.1 M glycine in PBS). Cells were lysed in cold RIPA buffer, dissolved in 2x LDS sample buffer and subjected to SDS-PAGE. Western blotting

**Antibodies.** Catalog numbers and dilutions used for Western blot; IF; immuno-fluorescence analysis; IP; immunoprecipitation; PLA, proximity ligation assay: NHE1-54 (Santa Cruz Biotechnology, sc-163239); WB and IF: 1:600; NHE1-53 (Millipore-Sigma, #AB9140); NHE1-53 (Millipore-Sigma, #C1956); PLA 1:2000; NHE1-53 (Millipore-Sigma, #C1956) and IgG isotype ctrl (Cell Signaling Technology, #2729) for ip 2 μg mg total protein; HFR-conjugated secondary antibodies (Agilent-Dako #PA0447 (mouse) and #PA0448 (rabbit)); WB: 1:4000; Alexa fluor-conjugated secondary antibodies (ThermoFisher #A10037 (mouse) and #A10042 (rabbit)); IF 1:600.

**Quantification and statistical analysis.** ITC measurements were repeated between 2 and 4 times; reported values are the average and standard deviation for the repeated measurements. Sigma Plot 12.1.3 was used for the statistical analysis of activity assays. For cell biological data, all data are shown as representative images or as mean measurements with standard error of means (SEM) error bars, and represent at least three independent experiments unless stated otherwise. A two-tailed paired Student's t-test was applied to test for statistically significant differences between two groups. When comparing more than two groups two-way analysis of variance (ANOVA) with Dunnett's multiple comparison post-test was used, except in Supplementary Fig. 2, where two-way ANOVA was applied. *$, **$ and ****$ denotes $p < 0.05, p < 0.01, p < 0.001$, and $p < 0.0001$, respectively.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability** All NMR chemical shifts have been deposited in the BioMagResBank with the accession codes BMRR 26755 and BMRR 27812. Atomic coordinates and structure factors have been deposited in the Protein Data Bank with the accession codes 6NUC, 6NUF and 6NUU. The source data underlying Figs. 2–c, 3b, 4b–g, 5b–h and Supplementary Tables 3 and 2 are provided as a Source Data file. All other data supporting the findings of this study are available from the corresponding authors on reasonable request.

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