Phosphorylation of conserved phosphoinositide binding pocket regulates sorting nexin membrane targeting

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Sorting nexins anchor trafficking machines to membranes by binding phospholipids. The paradigm of the superfamily is sorting nexin 3 (SNX3), which localizes to early endosomes by recognizing phosphatidylinositol 3-phosphate (PI3P) to initiate retromer-mediated segregation of cargoes to the trans-Golgi network (TGN). Here we report the solution structure of full length human SNX3, and show that PI3P recognition is accompanied by bilayer insertion of a proximal loop in its extended Phox homology (PX) domain. Phosphoinositide (PIP) binding is completely blocked by cancer-linked phosphorylation of a conserved serine beside the stereospecific PI3P pocket. This “PIP-stop” releases endosomal SNX3 to the cytosol, and reveals how protein kinases control membrane assemblies. It constitutes a widespread regulatory element found across the PX superfamily and throughout evolution including of fungi and plants. This illuminates the mechanism of a biological switch whereby structured PIP sites are phosphorylated to liberate protein machines from organelle surfaces.
ll endocytosed cargos are delivered to the early endosome, where they are sorted and transported to the plasma membrane, late endosome or TGN. The retromer relies on sorting nexin proteins in order to segregate the cargo proteins destined for the TGN or the plasma membrane. The sorting nexins associated with the retromer are distinguished by their architectures. The SNX3 subtype contains a PX domain but lack multimerization motifs, and may be found in vesicular, rather than tubular, carriers. The SNX1 subtype contains a Bin-Amphiphysin-Rvs (BAR) domain and form dimeric complexes capable of sensing and/or remodeling membrane curvature. How either type and their attached trafficking machines dynamically engage cellular membranes to control cargo trafficking remains unclear.

SNX3 is the best understood short sorting nexin that consists only of a single conserved PX domain and no flanking structural domains. Its cellular localization is principally determined by the specific interaction of its PX domain with PI3P, as presented at the cytosolic leaflet of the early endosome. Mutations within the conserved PI3P pocket of SNX3 abolish the membrane binding and compromise its endosomal functions. The binding of SNX3 to endosomes is a prerequisite for association with the retromer complex and positions the complex for cargo packaging and transits. The mechanism regulating this membrane complex assembly remains an unsolved mystery that is critical for the fidelity of subcellular trafficking. The recruitment of SNX3 to the cytosolic leaflet of the early endosome is a key determinant for productive retromer formation and it serves as a potential point for regulated binding and release. Visualizing how native SNX3 engages PI3P-containing membranes provides an opportunity to explain how retromer targeting and PX protein assemblies are controlled. As PI3P is constitutively present and abundant at the early endosome where a multitude of proteins recognize it, its level of production is unlikely to determine whether proteins membrane localize. Instead, we propose that another master regulatory switch must prevail, the identity of which remains unknown.

There are no structures showing sorting nexins inserted into membranes or in a regulated state. The structure of the PX domain of the distantly related yeast Grd19p protein has been reported, the human retromer structure has been solved, and coordinates of SNX3 and SNX12 PX domains coordinates have been deposited as 2YPS and 2CSK, respectively. However, key loops and terminal elements are missing in earlier structures, membrane binding data is lacking and the regulatory elements remain undefined. Moreover, no structure of any full-length sorting nexin interacting with a membrane mimic has been determined.

Here, we have characterized solution structures of native human SNX3 protein states, elucidating their interactions with each individual membrane component in order to better understand how membrane recognition is mediated and controlled. Membrane interaction by full length SNX3 is necessarily orchestrated by the specific recognition of the PI3P head group, followed by deep insertion of proximal hydrophobic elements into the bilayer. A conserved serine phosphorylation site at the rim of the PI3P binding site proved critical for switching off the interaction. Mutant forms mimicking the phosphorylated and non-phosphorylated states show that the interaction between SNX3 and early endosomal membrane is regulated in cells by Ser72 phosphorylation, which abolishes PI3P binding in vitro. This appears to constitute an all-or-none phosphorylation switch for dynamically regulating reversible endosome attachment for retromer assembly and detachment. Moreover, this highly structured regulatory element near the PIP docking site is shared with other sorting nexins that mediate membrane trafficking, suggesting a broad utility for controlling how proteins selectively recognize organelles and move cargo.

Results

Solution structure of human SNX3. In contrast to SNX-BAR proteins, which form oligomers, SNX3 is an obligate monomer. The full length SNX3 protein remains monomeric and monodispersed in physiological solutions, as does the closely related SNX12 PX domain based on the single peaks seen in analytical ultracentrifugation (AUC) experiments. Their sedimentation coefficients of 1.81 and 1.88 s for SNX3 and SNX12-PX, respectively, are consistent with molecular masses of 15–21 kDa for SNX3 and SNX12-PX, respectively (Supplementary Fig. 1a). This compares favorably with their theoretical molecular masses of 19.9 and 17.8 kDa. Narrow peak widths in 1H, 13N resolved NMR spectra (Supplementary Fig. 1b and ref. 11) are consistent with the SNX12 and SNX3 proteins being monomeric in solution, or when bound to PI3P molecules.

The structure of the 162 residue human SNX3 protein was elucidated under physiological solution conditions. The restraints used included 4984 distances derived from the volumes of crosspeaks in 15N-resolved and 13C-resolved nuclear overhauser enhancement (NOE) spectroscopy experiments, 188 backbone dihedral angles, and 88 hydrogen bond restraints (Table 1). The structure includes a classical PX fold preceded by 25 unstructured N-terminal residues and is followed by 16 residues exhibiting irregular structure based on the NMR data (Fig. 1 and Supplementary Fig. 1c). Conversely, the C-terminus is structured and interfaces with the α1, α3, and α4 helices via the Ile150, Tyr154, and Ile159 side chains, which interdigitate with the hydrophobic core of the PX domain. This juxtaposes the flexible N-terminus and structured C-terminus far from where the membrane is engaged, thus constituting a platform for tertiary interactions with the retromer alongside the 3-stranded β sheet (Fig. 1b), consistent with previous mutational studies. Hence this structure of functionally intact SNX3 reveals the entire protein’s structured elements and dynamic features, including those

| Table 1 Structural restraints used to calculate solution structures of SNX3 and its micelle complexes |
|-----------------------------------------------|
| **Experimental restraints**                   |
| NOE distance restraints                       |
| Unambiguous                                    | 3605 |
| Long range (|j-i| > 5)                       | 685  |
| Medium (4≤|j-i| ≤ 5)                       | 103  |
| Short (2 ≤ |j-i| ≤ 3)                       | 387  |
| Sequential                                    | 982  |
| Internal                                      | 1448 |
| Ambiguous                                     | 1379 |
| Hydrogen bond restraints                      | 44   |
| Dihedral constraints                          | 188  |
| Dihedral constraints                          | 94   |
| ψ                                            | 94   |
| ψ                                            | 94   |
| **Ambiguous distance restraints**             |
| Protein-micelle (PRES) (20 Å)                 |
| Asn100e, Phe103, Gly105, Asp107, Phe110       |
| PIP3-micelle (9.71 Å)                         |
| CB methyl group                               |
| Hydrogen bonds to PIP3                        |
| Arg70, Lys95, Arg118                         |
| Semi-flexible residues                        |
| Val39-Gly46, Lys95-Asp111                     |
| Interactions SNX3 to micelle                  |
| Hydrogen bonds (SNX3-micelle)                 |
| Arg43, Arg99, Gln100, Arg104                  |
| Non-bonded                                    |
| Arg43, Gly44, Lys95, Leu98-100, Pro102-Asp107 |

*Observed in at least 50% of the structural models
An additional helix connects PPII to complete with its C-terminal extension exhibiting a backbone bilayer based on analysis by the Membrane Optimal Docking β signifying, with acidic Glu30, Asp32, overwhelming negatively charged, with acidic Glu30, Asp32, Asp111, Asp112, Glu116, Glu117, and Glu123 residues contributing to SNX’s electronegative pole (Fig. 2) that would naturally orient away from the negatively charged membrane surface, simultaneously positioning the basic pole towards the phospholipid bilayer surface.

Nonspecific bilayer and specific PI3P interaction. The step-wise mechanism by which SNX3 recognizes phospholipids to initiate retromer assembly on the membrane was examined by NMR titrations of individual components. To study initial non-specific sampling of the membrane,14N-labeled SNX3 was titrated with either dodecylphosphocholine (DPC) or diheptanoyl phosphatidylcholine (DHPC) mixed with CHAPS at a 3:1 molar ratio. Similar chemical shift perturbation (CSP) patterns were observed with either mixed micelle (Fig. 2). In both cases, the perturbations mapped exactly onto the MIL, as delimited by residues Ala96 and Asp111. Both micelles were tested as the DHPC induces larger perturbations that were easier to resolve, and contains a more biologically representative headgroup, while DPC forms smaller micelles at lower concentrations that allow more complete

Fig. 1 Solution structure of human SNX3. a The structure of SNX3 closest to the mean is shown in two perspectives with regular secondary structures, including four α-helices (orange) and three anti-parallel β-strands (blue). The segment delimited by α1 and α2-helices contains a polyproline type II helix (PPII) and α helical elements by the membrane inserting loop (MIL). The C-terminal segment is highlighted in magenta. b The ensemble of structures is represented as a sausage representation of the backbone traces, with the thickness and color being related to the pairwise r.m.s. deviation up to a cut-off at 3 Å (see inset scale). c Structure-based alignment of the SNX3 PX sequences. The C-terminally extended PX domains of SNX3 proteins from Homo sapiens (Hs), Caenorhabditis elegans (Ce), Drosophila melanogaster (Dm), Danio rerio (Dr), Saccharomyces cerevisiae (Sc, also known as Grid1p), and Neurospora crassa (Nc), as well as SNX1 from Arabidopsis thaliana (At), as there is no SNX3 homolog in this species, were aligned with ClustalW. Absolutely conserved, identical, and similar residues are shaded in brown, green, and yellow, respectively. The residues corresponding to Hs SNX3 Ser72 are magenta, and those that contact PI3P are indicated with a red asterisk. The positions of helices and extended elements are underlined and labeled.
saturation of the complex. Large perturbations identified the key residues involved in the interaction as being Leu98, Gln100, Phe103-Gly105, and Ile109, which present exposed sidechains for residues involved in the interaction as being Leu98, Gln100, Phe103-Gly105, and Ile109, which present exposed sidechains for.

**Table 2 Structural statistics for the solution structures of SNX3 and its micelle complexes**

| Structure statistics | Values |
|----------------------|--------|
| Residual experimental violations | 0 |
| NOE < 0.5 Å | 0 |
| NOE > 0.3 Å | 4 |
| Dihedral restraints >5 degrees | 0 |
| Energies (kcal mol⁻¹) | |
| E_RMSD | 262.05 ± 12.13 |
| E_diss | 3.48 ± 0.94 |
| E_bound | 64.20 ± 3.47 |
| E_improper | 138.10 ± 9.25 |
| E_angle | 278.56 ± 11.19 |
| E_dih | -314.5 ± 29.42 |
| E_dige | 918.78 ± 8.42 |

**P13P anchors SNX3 deep into the bilayer.** The ternary complex was assembled by progressive addition of P13P molecules and micelles to SNX3. Assignment of the complex involved titrating Ins(1,3)P₂ to levels approaching saturation of the protein:micelle complex. This allowed tracking of the resolved ¹H,¹³N signals (Supplementary Fig. 2a) and comparison with the slow exchange binding data obtained with c₄ and c₈-P13P ligands (Supplementary Fig. 2b). Perturbations seen in Arg70, Ser72, Leu92, Arg99, Phe103, Gly105, and Gln100C resonances of the ternary complex mirrored those previously observed with micelles or P13P derivatives alone. The similar patterns of perturbations induced by the individual and combined components show that the P13P pocket and MIL interactions are local and could conceivably provide complementary effects on membrane specificity and affinity, respectively.

In order to structurally characterize the ternary complex, the exchange kinetics were tuned by varying the length of the P13P acyl chains. The presence of zero, short or medium length chains progressively decreases the ligand exchange rates, with c₄-P13P titration into micelle-saturated SNX3 exhibiting a slower off rate and higher affinity than the headgroup alone (Fig. 2, Supplementary Figs. 2b & 3). This reflects the stabilization provided by insertion of its acyl chains into the micelle in parallel with the headgroup binding to the SNX3 basic pocket. Ternary complex restraints were obtained from ligand titrations with DHPC and DPC-based while collecting¹⁵N-resolved and¹³C-resolved spectra with cryoprobe-equipped 800 and 900 MHz NMR spectrometers. The orientation of SNX3 on P13P-containing micelles was defined using the intense¹³C signals, particularly from interfacial methyl groups. The resonances of the ligand-bound protein were assigned from c₄-P13P titrations, yielding ¹³C-resolved intermolecular NOE distances between Lys95 and H4 and H6 of P13P, between Ile109 and H1 and H3, and between Arg118 and H4, thus defining the headgroup orientation (Fig. 2b). The slowly exchanging SNX3:micelle complex formed using c₄-P13P allowed resolution of the bound and free state peaks, with introduction of 5-doxyl PC broadening the signals of MIL residues Phe103, Gly105 and Asp107 and Phe110 (Fig. 3a) and Supplementary Fig. 2c) compared to non-paramagnetic controls. Thus these SNX3 elements insert deeply into P13P-loaded micelles, in contrast with PC-only micelles. Hence, SNX3 relies on P13P to anchor stably into endosomal membranes, while P13P’s absence leaves SNX3 free to dislodge after briefly sampling the bilayer. Comparison of the NOE patterns between the free and mixed micelle-bound SNX3 states indicated no major conformational changes, as corroborated by their similar circular dichroism (CD) spectra (Supplementary Fig. 1d) and local CSPs observed for SNX3’s states (Fig. 2, Supplementary Figs. 2 & 3). Together this yielded all the CSP, PRE, and NOE restraints needed to dock SNX3’s structure to the single P13P and 54 DPC molecules within the micelle model (Table 1) in order to define how such sorting nexins structurally recognize the endosome surface.
Structural basis of membrane recognition by SNX3. How SNX3 orients itself onto membrane surfaces to anchor retromers was calculated stepwise using NMR restraints. First, the c8-PI3P headgroup was docked into its binding pocket using intermolecular distances, chemical shift changes and co-crystal contacts. This showed a canonical ligand orientation, with Arg70, Lys95, and Arg118 residues forming hydrogen bonds with the inositol phosphate and hydroxyl groups, and Ile109 contacting the ring (Fig. 3b, c). The structure of SNX3 bound to C8-PI3P and micelles, as calculated by HADDOCK (Fig. 3d), showed that SNX3 inserts deeply, with only 33.7 Å separating the centers of the micelle and PX domain, as opposed to the 38.7 Å separation seen in the Vam7p:micelle complex. This large interface buries 3404 ± 262 Å² and is defined by a higher density of intermolecular contacts.

Table 3 Affinities of SNX3 for lipid molecules and mixed micelles

| Ligand                     | K_d (μM)   |
|----------------------------|------------|
| Ins(1,3)P_2                | 169.6 ± 34.1 |
| c4-PI3P                    | 158.9 ± 36.1 |
| DHPC + CHAPS               | >2000      |
| DPC + CHAPS + Ins(1,3)P_2  | 57.4 ± 16.4 |
| DPC + CHAPS + c4-PI3P      | n.d. (slow exchange) |
| DHPC + CHAPS + c4-PI3P     | n.d. (slow exchange) |
| DPC + CHAPS + c8-PI3P      | n.d. (slow exchange) |

Values determined by NMR chemical shift perturbations.

Fig. 2 Membrane docking states of SNX3. The electrostatic surface potential of the lipid-free structure is colored blue and red for positive and negative charges, respectively, as calculated with PROPKA settings at pH 7 and APBS, and oriented as in Fig. 1a in the top left. The PI3P binding pocket and affinity were shown by HSQC ligand titrations, with the SNX3 ribbon colored according to the extent of H and 15N, CSPs by addition of 4.1 fold excess c4-PI3P, as drawn in magenta (top, right). The site of nonspecific micelle association is shown by the surface of the PI3P-free PX domain (below, left) colored on the basis of absolute CSPs induced by addition of 160-fold excess DPC. The position of the lipid bilayer is shown in green. The site of stable micelle association by the PI3P-bound PX domain is shown by the ribbon structure at the lower right, which is colored according to the absolute CSPs induced by addition of c4-PI3P to 20-fold DPC excess. The respective affinities based on the NMR titrations are indicated, and residues whose resonances show large, medium, small and no CSPs are colored red, orange, yellow, and white, respectively.
restraints than has been measured for other PX domains\textsuperscript{10,15,16} (Fig. 3d; Table 1). The SNX3 MIL is particularly long, with residues including Arg99, Gln100 or Arg104 exhibiting contacts with PI3P and seven PC molecules to orient the protein on the membrane in such a way that its β-sheet remains accessible to the retromer complex\textsuperscript{6}. The extensive PI3P-micelle interface also includes the β1-β2 hairpin loop, with Arg43 making electrostatic contacts with PC headgroups, the specific ligand interactions of the α1 and α2 residues, and bilayer insertion of the PI3P acyl tails. Together this yields a unique insertion angle of 31.0° ± 5.6° for the protein’s long axis into the micelle interior (Table 2) using established protocols\textsuperscript{14}. The conservation of the interfacial residues across the superfamily (Fig. 4) along with MODA-based identification of the respective membrane interaction surfaces\textsuperscript{12} in each available PX domain structure indicates that other sorting nexins employ similar membrane binding modes.

PI3P site phosphorylation blocks localization. A potential mechanism of regulation of SNX3’s fast and slow release from

Fig. 3 Model of SNX3 associated bound to micelles. a The experimentally driven molecular docking was based on the interaction of SNX3 with individual components, measuring CSPs for micelles (top) and c4-PI3P (middle). Asterisks mark residues with substantially broadened NMR lines. The membrane inserting residues were identified from PREs (bottom) obtained by adding 5 doxyl PC to 32 mM DHPC:CHAPS micelles in the absence (black) or presence of c8-PI3P (gray). b Strips extracted from the \( ^1H\)-\( ^1H \) planes of \( ^13C \)-edited HSQC-NOESY spectra and corresponding to Ile109’s \( ^1H \)-\( ^13C \) resonances are compared for SNX3 bound to micelles and c4-PI3P (red) or ligand-free (black). The overlaid \( ^13C \)-HSQC and \( ^1H \)-\( ^1H \) TOCSY spectra are shown alongside the DHPC: CHAPS peaks (green). c The interactions between PI3P and SNX3 residues are depicted. The recognized non-exchangeable inositol protons are color-coded to match the interacting residue. Conserved hydrogen bonds observed in PX: PI3P structures are represented by dotted lines. d Docked structure of the lowest energy complex between SNX3, PI3P and micelles. Residues active during the docking are indicated on SNX3. Significant CSPs for for Ins(1,3)P\textsubscript{2} (orange) and/or micelles (yellow) are shown. Those involved in intermolecular interactions with PI3P (red) or micelles insertion (blue) are also displayed. The phosphate groups of PI3P are indicated by P1 and P3.
weak and strong interactions with bilayers that are either PI3P-free or loaded, respectively, was investigated. We proposed that the conserved Ser72 position (Fig. 4), which is situated near the PI3P-binding Arg70 residue (Fig. 5a & Supplementary Fig. 4), could play a crucial role. Situated at the start of α1, Ser72 is solvent-exposed, helical in structure and environmentally hypersensitive, exhibiting NMR signal variations when the pH or buffer changes. The role of Ser72 was of interest as it is predicted to be phosphorylated[17] as evidenced in various tissues[18-20] and confirmed by mass spectrometry analysis of purified SNX3 purified from HeLa cells (Supplementary Fig. 5). The SNX3 structure indicates that phosphorylation here would prevent ligand binding by blocking inositol 3-phosphate entry and negating the optimal membrane docking propensity here.

To test this PIP-stop hypothesis, SNX3 mutants mimicking constitutively non-phosphorylated (S72A) and phosphorylated (S72E) states were designed and confirmed as folded by CD (Supplementary Fig. 1d). Compared to the wild-type form, selective membrane binding activity was retained by S72A mutant but abolished by the S72E mutant (Fig. 5b, Supplementary Fig. 6). The S72E mutant is expected to compete efficiently for the SNX3 binding site on the membrane, whereas the S72A mutant does not bind efficiently. Therefore, the phosphorylation status of Ser72 may play an essential role in determining the interaction of SNX3 with PI3P and the membrane.

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Discussion

Binding of sorting nexins to endosomal membranes leads to recruitment of retromers destined to the TGN and multivesicular body genesis. To understand this mechanism and its regulation, structures of SNX3 states were solved, illuminating how weak sampling and tight anchoring of bilayer surfaces occur. Membrane recognition includes a long MIL which includes an extra helix between the PPII motif and q2 that mediates both superficial, non-specific interaction with phospholipid surfaces as well as deep hydrophobic insertion into bilayers. An adjacent pocket recognizes P13P and acts as the principal anchor, which leads to interfacial engagement of the β-α1 loop and the beginning of q2 to form a strong membrane attachment and unique angular positioning on the surface of endosomes.

The structural characterization of the complete SNX3 protein in free, lipid-bound, micelle-inserted and mutated states exposes recurrent functional and regulatory features. The complete membrane interface of a sorting nexin has been elucidated, showing how a unique MIL element shared with SNX1, SNX2, SNX4 and SNX12 (Fig. 4) engages bilayers broadly to mediate endosomal sorting. Of these proteins SNX12 appears most like SNX3, while in SNX1 and SNX2 the hydrophobic and cationic character of the MIL is enhanced, perhaps endowing them with unique phospholipid binding modes including potential PI(3,5)P2 binding. A pair of unusual sorting nexins, SNX5 and SNX6, differ in their lipid binding motifs and modes, and bind to PI(4,5) P2 and PI4P, respectively.

An unusual feature seen in SNX3’s solution structure is the extended C-terminal element that juxtaposes the termini. Although C-terminal extensions pack into other SNX structures, they generally involve α-helical segments as in SNX1 and SNX3 (pdb 4AKV), rather than the extended segment found in SNX3 as well as SNX1, SNX12 and SNX14. This forms part of the retromer docking surface that allows positioning a defined distance off the membrane surface (Fig. 7).

Attachment of a phosphate to a structured P13P site was found to compete directly with phospholipid ligand binding. This PIP-stop could control membrane binding of a sorting nexin molecule or an entire retromer. Whether this event regulates the formation, cycling or disassembly of such complexes remains to be determined. Moreover, the enzymes that create PIP-stops remain a mystery, as protein kinases are usually thought to add phosphates to induce conformational changes or create protein binding sites. Here conformational changes or other proteins are not required, the PIP-stop simply repels negatively charged lipid bilayers. This opens new perspectives for understanding how protein association with membrane targets are controlled in cellular systems. Indeed phosphates are added to the PIP binding sites of diverse sorting nexins, revealing a widespread mechanism that is conserved across most of the SNX superfamily. This post-translational modification abolishes membrane binding not only by SNX3 but also by other sorting nexins including SNX1 and SNX12, suggesting a common mechanism for controlling protein assembly and disassembly on membranes.

Our results suggest that the identity of the residue corresponding to SNX3 Ser72 could play a determining role in the specific recognition of different organelle membranes. For instance, the PX domains of SNX5 and SNX6 have glutamates at this position and are reported to recognize PI4P and PI(4,5)P2 lipid, which are concentrated in the trans-Golgi network and plasma membranes, respectively. In contrast SNX14 has a leucine here, displays cytoplasmic localization and lacks 3-phosphoinositide binding. Thus the PIP-stop residues also could contribute to the PIP specificity and subcellular membrane destination of PX superfamily members.

Figs. 6c, d). This indicates that introducing a negative charge here prevents P13P recognition, presumably by compromising the Arg0-mediated contacts. Moreover, phosphorylation of Ser72 specifically blocks the protein from endosome docking, as validated in cellular assays. In particular, the non-interactive SNX3S72E mutant is cytosolic, much like the R70A mutant that cannot bind membranes because of a defective P13P-binding site, whereas the constitutively membrane-active SNX3S72A mutant is distributed on endosomes like the wild type protein (Fig. 6). We previously showed that SNX3 overexpression inhibits endosome maturation and delays the degradation of endocytosed EGF receptor. Consistent with these findings, expression of SNX3S72E, like the WT protein, significantly delayed EGF receptor degradation, when compared to neighboring untransformed cells (Fig. 6a, c, d; Supplementary Figs. 7, 9). By contrast, the S72E mutant had essentially no effect on EGF receptor degradation, like SNX3R70A or free GFP (Fig. 6a, c, d). The submicromolar affinity and high specificity for P13P-containing bilayers would retain the SNX3 protein by endosomal membranes, with its membrane-embedded Ser72 and slow off-rate leaving limited room for release. Once freed, if only transiently, the membrane-embedded Ser72 and slow off-rate leaves limited room for release. Once freed, if only transiently, the membrane-embedded Ser72 and slow off-rate leaves limited room for release. Once freed, if only transiently, the membrane-embedded Ser72 and slow off-rate leaves limited room for release. Once freed, if only transiently, the membrane-embedded Ser72 and slow off-rate leaves limited room for release.

The PIP-stop motif is highly conserved across SNX3 homologs in plants, fungi, invertebrates and vertebrates, as well as across much of the sorting nexin superfamily (Figs. 1c and 4). Indeed, phosphorylations of the corresponding serines have been identified in proteins including SNX1 and SNX12. To test their biological impacts, mutations of the corresponding serine residues were investigated. As predicted, the SNX1S188E mutant distribution was cytosolic in contrast to the endocytic localization of the wild-type form, while the control SNX1S72A mutant retained the wild-type punctate pattern of wild-type SNX12 (Supplementary Figs. 8, 10). Thus, they mirrored the SNX3 pattern, consistent with parallel regulatory functions. Hence, the PIP-stop mechanism appears to constitute a common way of controlling sorting nexin function, and infers a general way to modulate membrane attachment by selective phosphorylation of structured PIP binding sites.
Diverse species consistently show the importance of phosphorylation of SNX3 Ser72 or its equivalent phosphorylatable residue. In Drosophila SNX3 this is the primary phosphorylated site within the entire protein. In budding yeast the homologous serine is the sole phosphorylated residue in the full length protein. Indeed, selective phosphorylation of SNX3 Ser72 (or its homologous residue) is particularly conserved throughout evolution. We propose that the context of the PIP-stop, containing the EGF receptor after 15 min was quantified per cell (b) and is compared to the total intensity of the GFP fluorescence signal per cell (c). The experiments were as in a for the indicated time periods. The total EGF receptor per cell was quantified in transfected cells (solid line) and in neighboring untransfected cells (dashed line) from the same well of the 96-well plates. The integrated intensity is expressed as a percentage of the values observed at 15 min. Approximately 1000 cells were analysed per condition in n = 3 independent experiments. Error bars indicate SEM. Statistical significance is calculated using one-way ANOVA analysis with Bonferroni’s post-test. Levels of significance are indicated as follows: *p < 0.05; **p < 0.001

Methods

Protein expression and purification. A sequence encoding full length human SNX3 was synthesized (Genescript), cloned into a pET45b vector (Novagen) and overexpressed in E. coli BL21(DE3) cells (Novagen) as a N-terminally His6-tagged protein. Bacterial cultures were grown in Luria-Bertani broth or M9 minimum media supplemented with 15N-H4Cl and 2H-labeled 13C-labeled, or 13C-labeled glucose at 37 °C until an OD600 of 0.6. After addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), the protein was expressed for 16 h at 18 °C. Cells were harvested by centrifugation (6000g, 20 min) and resuspended in 20 mM Tris pH 7.5, 100 mM NaCl, 20 mM imidazole, 1 mM NaN3 and 1 mM DTT. The cells were lysed with an Emulsiflex (Avastin) and the soluble protein was purified over a Ni2+–NTA affinity column (GE Healthcare). Fractions containing SNX3 were pooled and applied a Superdex-75 (GE Healthcare) size exclusion column and eluted with 20 mM sodium phosphate buffer pH 6.5, 100 mM NaCl 1 mM DTT. The PX domain of SNX12 (SNX12-PX) was expressed in a pET45b vector and purified as with SNX3, except for the cleavage of the N-terminal histidine tag at an Enterokinase site prior to size exclusion chromatography. Full length SNX7 was cloned into a pGEX-5X vector and the GST-tagged protein was purified using a GST-trap column (GE Healthcare), cleaved with Factor Xa and purified over a Superdex-200 column (GE Healthcare). DNA sequences are shown in Supplementary Table 1. Mutations were generated with QuikChange Lightening (Stratagene) and verified by DNA sequencing.

NMR and structure calculation. NMR spectra of 15N, 13N,15C, and 2H/13N/13C labeled protein were acquired at 298 K on 600 and 800 MHz Varian Inova spectrometers equipped with 5 mm cryogenic probes. The spectra were processed with NMRpipe and analysed with CCPNMR. The backbone ^H, 13N, and 13C resonances were sequentially assigned using standard 3D experiments. Distance restraints were derived from 15N-edited NOEY-HSQC and 13C-edited NOESY-HSQC experiments (Tmix = 100 ms) optimized for aromatic or aliphatic groups.
membrane and retromer recruitment. A fraction of free SNX3 can be phosphorylated at Ser72, which prevents membrane re-binding until phosphorylation occurs to allow tight anchoring into the endosomal membrane and retromer recruitment. Model of the retromer assembly on a membrane represented by DMPC bilayer, with the solution structure of SNX3 bound to PI3P (red, with green C-terminus) superimposed on the crystal structure of SNX3 (blue) and attached Vps26 (silver-green) and Vps35 (silver-red) structures depicted.

Dihedral angle restraints were derived from DANGLE42. Hydrogen bond restraints were applied to orient the acyl chains, for rigid body insertion of the SNX3:PI3P complex into micelles47 and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) bilayer48 models. Distances between the methyls of PI3P were restrained to 11.4 Å based on dioleoyl-PC (DOPC) bilayer models to enforce realistic conformations. Distances from the center of the micelle to the acyl chain methyl were set to 9.71, and to 21.12 Å for the glycerol C3 atom, which corresponds to the average distance with the C8 and C1 atoms of DPC in micelles49. To handle the high temperature dynamics, the distances between guanidinium group of Arg118 and H4 and H5 atoms of PI3P were set to 3 Å. Molecular dynamics were applied to orient the acyl chains, for rigid body insertion of the SNX3:PI3P complex into micelles and then relaxation of the system. The docking protocol yielded 200 structures which were refined in explicit water and ranked according to their energies.

Analytical ultracentrifugation. The SNX3 and SNX12-PI3P samples were prepared in the buffer used for NMR experiments and their monomeric states were evidenced using a XL-1 analytical ultracentrifuge (Beckman Coulter). The sedimentation coefficient (s) and distribution [c(s)] were determined from sedimentation velocity experiments with a two-sector cell at 129,024 x g for 17 h at 4 °C. The absorbance of the sample was measured at a wavelength of 280 nm throughout the cell. The partial specific volume of the sample, the viscosity and density of the buffer were calculated in SEDNTERP and used with the coefficient of sedimentation c(s) in the routine SEDFIT49, with approximate molecular weights of the species in solution being deduced from the simplified model of continuous c (M) distribution.

Liposome binding. In order to assay lipid interactions POPC and PI3P (98:2 mol: mol) were mixed in chloroform and dried under a nitrogen flux and under high vacuum. Lipid mixtures (2 mM) were obtained by resuspension of the lipids into sodium phosphate 20 mM pH 7, 100 mM NaCl from which 75 µL were mixed with 25 µL of SNX3 (8 µM). After incubating the mixture for 10 min at 25 °C, the liposomes were pelleted, washed three times with the resuspension buffer and loaded on SDS-PAGE gels and bands for quantification by densitometry (Syngene). Liposomes (0.5 mM lipid) were prepared for surface plasmon resonance (SPR) detection in 50 mM Heps pH 7.2, 50 mM NaCl. Unilamellar vesicles were produced by ten freeze and thaw cycles at 25 °C using liquid nitrogen, followed by extrusion of the vesicle through a 0.1 µm (Avanti) polycarbonate membrane. SPR measurements were carried out on a Biacore 3000 instrument (GE Healthcare). Vesicles were coated on a L1 sensor chip (GE Healthcare) at 5 µL min⁻¹. A reference lane coated with POPC alone was used to measure the specific response for lanes coated with PI3P. Equilibrium measurements were obtained by injecting 85 µL of protein at 3–5 µL min⁻¹. For kinetic measurements, the flow was increased to 30 µL min⁻¹ and 40 µL of protein was injected. Surfaces were regenerated by injections of 10 µL of 10 mM NaOH at 100 µL min⁻¹.

Cell culture and transfection. HeLa-MZ cells, which were provided by Marino Zerial (MPI-CBG, Dresden), and HeLa43 cells were maintained as described. HeLa cells are not on the list of commonly misidentified cell lines maintained by the International Cell Line Authentication Committee. Our HeLa-MZ cells were authenticated by Microsynth (CH), which revealed 100 % identity to the DNA profile of the cell line HeLa (ATCC® CCL-2®) and 100 % identity over all 15 autosomal STRs to the Microsynth’s reference DNA profile of HeLa. Cells are mycoplasma negative as tested by GATC Biotech (Germany). Mutations were introduced in wt SNX3-GFP by QuikChange site-directed mutagenesis (Stratagene) using as primers: R78A, 5′-gaaggtagtagagcaacctctctctcagtcgta-3′ and 5′-ctacttcgacaagtctatcgtcgcag-3′; R72A, 5′-gaaggtagtagagcaacctctctctcagtcgta-3′ and 5′-tacttcgacaagtctatcgtcgcag-3′; R75A, 5′-gaaggtagtagagcaacctctctctcagtcgta-3′ and 5′-tacttcgacaagtctatcgtcgcag-3′; R72A′, 5′-gaaggtagtagagcaacctctctctcagtcgta-3′ and 5′-tacttcgacaagtctatcgtcgcag-3′. Cells were transfected transiently with plasmid DNA using FuGENE HD (Promega) or TransIT-X2 Dynamic Delivery System (Mirus) according to the manufacturer’s recommendations.

Light microscopy. HeLa-MZ transfected with wt SNX3-GFP or the indicated mutants were starved for 4 h and then incubated in the presence of EGF (100 ng mL⁻¹) for the indicated time periods. Cells were then fixed and stained with antibodies to the EGF receptor, followed by fluorescently labeled secondary antibodies. Images were recorded using a Carl Zeiss AxioObserver microscope. The cell images were analyzed with ImageJ software. Proteins were quantified by immunoblotting.
UV circular dichroism. Proteins were dialyzed against 10 mM potassium phosphate buffer, pH 7.2. Data were acquired on a Jasco J-810 spectropolarimeter using a HELLMAX cuvette with an optical path length of 0.2 mm. Signals were obtained by averaging 8 scans measured between 190 and 260 nm with a 0.2 nm increment at a sampling rate of 50 nm min⁻¹.

Mass spectrometry. HeLa M7 cells at 60–70% confluency were transiently transfected with indicated GFP constructs: 18 h later cells were scraped in lysis buffer [30 mM Tris–HCl pH 7.4, 1 mM EDTA, 0.5% NP40 and Protease/Pherase Inhibitor Cocktail (CST)] and lysed. GFP-Trap MA beads (Chromotek) were used for GFP precipitation. After 45 min incubation at 4 °C with the cleared cell lysates, beads were washed with lysis buffer without detergents. Proteins attached to the beads were eluted by incubating the beads at 95 °C in 2× Laemmli buffer, and subjected to gel electrophoresis and western blotting.

Data availability. SNX3’s coordinates have been deposited in the Protein Data Bank, with accession code 2MXC, and its NMR chemical shifts are available in BMRB Entry 25402. The authors declare that all other data supporting the findings of this study are available in the paper and its supplementary information files or from the corresponding author upon reasonable request.

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**Author contributions**

M.L. performed NMR and biochemical experiments on sorting nexins, as well as structure calculations. S.R. and J.K. produced constructs and proteins. C.U. and D.M. performed cell biology experiments. M.L., C.U., J.G., and M.O. designed experiments. M.L., C.U., J.G., and M.O. wrote the paper.

**Additional information**

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