Structural determinants controlling 14-3-3 recruitment to the endocytic adaptor Numb and dissociation of the Numb/α-adaptin complex

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ABSTRACT
Traffic of cargo across membranes helps establish, maintain, and reorganize distinct cellular compartments and is fundamental to many metabolic processes. The cargo-selective endocytic adaptor Numb participates in clathrin-dependent endocytosis by attaching cargoes to the clathrin adaptor α-adaptin. The phosphorylation of Numb at Ser-265 and Ser-284 recruits the regulatory protein 14-3-3, accompanied by the dissociation of Numb from α-adaptin and Numb’s translocation from the cortical membrane to the cytosol. However, the molecular mechanisms underlying the Numb/α-adaptin interaction and its regulation by Numb phosphorylation and 14-3-3 recruitment remain poorly understood. Here, biochemical and structural analyses of the Numb/14-3-3 complex revealed that Numb phosphorylation at both Ser-265 and Ser-284 is required for Numb’s efficient interaction with 14-3-3. We also discovered that an RQFRF motif surrounding Ser-265 in Numb functions together with the canonical C-terminal DPF motif, required for Numb’s interaction with α-adaptin, to form a stable complex with α-adaptin. Of note, we provide evidence that the phosphorylation-induced binding of 14-3-3 to Numb directly competes with the binding of α-adaptin to Numb. Our findings suggest a potential mechanism governing the dynamic assembly of Numb with α-adaptin or 14-3-3. This dual-site recognition of Numb by α-adaptin may have implications for other α-adaptin targets. We propose that the newly identified α-adaptin–binding site surrounding Ser-265 in Numb functions as a triggering mechanism for the dynamic dissociation of the Numb/α-adaptin complex.

Membrane traffic establishes, maintains, and reorganizes distinct compartments of a cell while retaining the compositional (proteins, lipids, etc.) and functional heterogeneity of the donor and acceptor membranes. Clathrin-coated pits/vesicles (CCPs/CCVs), the major membrane traffic carriers, mediate many post-Golgi trafficking routes including internalization and recycling via clathrin-mediated endocytosis, which is fundamental to cell nutrition, neurotransmission, and cellular signaling (1-3). The Adaptor protein 2 (AP2) complex is the most abundant clathrin adaptor. It forms a large globular heterotetrameric (αβ2-μ2-σ2) core structure with two appendage domains (from the C-terminal domains of the α and β2 subunits), and plays important roles in many vesicle...
Structural basis of Numb binding to α-adaptin and 14-3-3 trafficking pathways within the cell. The α (also called α-adaptin) and β2 subunits mediate the binding to the target membrane and the recruitment of clathrin, respectively, whereas the µ2 and σ2 subunits recognize the Yxxφ (where x represents any amino acid and φ indicates a hydrophobic residue hereafter) and di-leucine (diLeu) motifs on cargo proteins, respectively (4,5). The appendage domain of α-adaptin (referred to as α-appendage hereafter) is also responsible for recruiting a large number of accessory/regulatory proteins, including Eps15, by binding to DPF motifs within these otherwise very different proteins (6-8). Two conserved target binding sites have been identified on α-appendage, namely, the “top” site that recognizes the DPF/FxFxF/FxxFxxL motifs and the “side” site that binds to the WVxF/W motif (6,7,9).

The evolutionarily conserved cell fate determinant Numb displays a complex pattern of functions in asymmetric cell division, cell adhesion, cell migration, endocytosis, and ubiquitination of specific substrates within a number of signaling pathways, and it acts as a tumor suppressor in certain cancers (10-17). As a cargo-selective endocytic adaptor, Numb regulates both clathrin-dependent and independent intracellular trafficking of multiple molecules including Notch, integrin, and Rab7 (18-20). Additionally, Numb has been demonstrated to regulate the homotypic fusion of early endosomes during intracellular trafficking (21). From the N- to C-terminus, Numb contains a phosphotyrosine binding (PTB) domain, a proline-rich region, two DPF motifs and an NPF motif. The PTB domain of Numb can bind to the intracellular domains of trans-membrane proteins and thus promotes their recruitment into CCPs/CCVs and their subsequent internalization through AP2 (18,22). The NPF motif is important for binding to Eps15 homology (EH) domain-containing proteins, e.g., Eps15 (23). The carboxyl terminal DPF motif of Numb mediates a conserved interaction with α-adaptin of the AP2 complex (22-26). In HeLa cells, Numb was found to be colocalized with AP2 at substratum plasma membrane punctate and cortical membrane-associated vesicles (26,27). It was recently shown that two conserved Ser residues (S265 and S284 in mouse Numb) in the central region of Numb could be phosphorylated by Ca²⁺/calmodulin-dependent protein kinase I (CaM-KI) (25) and atypical protein kinase C (aPKC) (19,26,27) both in vivo and in vitro. Moreover, aPKC-dependent phosphorylation of Numb leads to the translocation of Numb from the cortical membrane to the cytosol (26,27). A potential mechanism is the phosphorylation-induced recruitment of 14-3-3, which coincides with the disassociation of AP2 from Numb, suggesting that 14-3-3 binding to Numb may regulate its association with AP2 (28).

14-3-3 proteins are important regulatory proteins that modulate an enormous and diverse group of physiological processes (29-32) and are thus linked to plenty of diseases such as cancers (33). 14-3-3 family proteins are phosphoserine/threonine (pS/T) binding modules that recognize proteins with consensus sequences RSxpSxP (mode 1), RxφxpSxP (mode 2) and p[S/T]x-COOH (mode 3), thereby modulating their target binding properties, controlling their subcellular localization, as well as constraining their protein conformation (34,35). 14-3-3 can also bind to unphosphorylated targets (36) or prenyl-phosphorylation motifs (37), generating a diverse array of over 200 substrates (32). Owing to their dimeric nature, 14-3-3 can interact with dimeric single-site targets or two-site monomeric targets (34,36,38,39), and the two-site phospho-partners normally have enhanced binding affinities towards 14-3-3 compared with the single-site ones. Due to the lack of detailed structural information, it is not clear how 14-3-3 interacts with Numb upon its phosphorylation, whether both pS265 and pS284 sites are required for efficient Numb/14-3-3 interaction, and how this binding interferes with Numb/AP2 interaction.

Here we solved the crystal structure of 14-3-3 in complex with di-phosphorylated Numb (pS265S284, referred to as pSS hereafter; Fig. 1A) peptide, providing an atomic-level picture for the site-specific interaction between Numb and 14-3-3. Next, we discovered another Numb binding site on α-appendage distinct from the well-known DPF motif binding “top” site. We further found that these two weak Numb binding sites function cooperatively in binding to Numb with high affinity. Structural analysis...
of the Numb/14-3-3 and Numb/α-appendage interactions suggested that phosphorylation-induced 14-3-3 binding would impair the dual-site Numb/α-appendage interaction, resulting in dissociation. In addition to the potential role of 14-3-3 in regulating Numb/α-adaptin-mediated endocytosis, our study also provides a new dual-site target binding mode for α-adaptin.

Results

Phosphorylation of Numb at S265 and S284 recruits 14-3-3

Sequence analysis of mouse Numb revealed that neither the S265 nor the S284 sites is a canonical RSxpSxP or RxφxpSxP 14-3-3 binding motifs (Fig. 1A). To understand how the aPKC/CaM-KI-mediated phosphorylation of Numb at S265 and S284 recruits 14-3-3 (25,28), we assayed the interaction between 14-3-3η and synthetic Numb phospho-peptides (Fig. 1A). Isothermal titration calorimetry (ITC) experiments showed that neither single phosphopeptides (pS265 or pS284) showed detectable binding to 14-3-3η (Fig. 1B). In contrast, the di-phosphorylated pSS peptide bound to 14-3-3η with high affinity (Kd~ 0.32 µM; Fig. 1B), suggesting that both pS265 and pS284 fragments of Numb function synergistically in interacting with 14-3-3η. In line with the ITC results, the cell lysate GST pull-down assay showed that only the wild-type (WT) Numb could be robustly pulled down by GST-tagged 14-3-3η (Fig. 1C). The S265A mutation dramatically reduced Numb/14-3-3 interaction, whereas the S284A mutation and the S265,S284A double mutation completely disrupted this interaction. This data is consistent with the results of a previous brain extract GST pull-down assay (28).

Overall structure of the phospho-Numb/14-3-3 complex

To elucidate the structural basis of the cooperative binding between the di-phosphorylated Numb peptide and 14-3-3, we determined the crystal structure of 14-3-3η in complex with the synthetic 30-amino-acid pSS peptide at 2.1 Å resolution (Fig. 2A, 2B and Table 1). The electron density map allowed the building of 21 residues of the peptide, clearly illustrating both primary interaction sites. In the pS265 and pS284 sites, residues 262-267 and 271-287 were visible, respectively (Fig. 2C). The other amino acids in the peptide could not be traced, presumably due to its intrinsic disorder and lack of contacts with 14-3-3η. In the complex structure, one Numb pSS peptide asymmetrically interacts with a 14-3-3η homodimer to form a trimeric architecture (Fig. 2A and 2B). In line with the crystal structure, ITC data revealed that this peptide bound to a 14-3-3 dimer with 1:1 stoichiometry (N= 1, Fig. 1B). Each monomeric 14-3-3η subunit consisted of nine antiparallel α-helices, and residues within the first four α-helices formed the dimer interface as well as the floor of the channel (Fig. 2A). Though neither the pS265 nor pS284 fragment of Numb was the canonical RSxpSxP or RxφxpSxP motif, the Numb residues resided along the same amphipathic groove between the sidewall and floor of the channel of 14-3-3 in an extended conformation, just as the canonical 14-3-3 binding motifs do (34) (Fig. 2A and 2B). Polar contacts were found between main chain amides from Numb pSS and side chains from residues in 14-3-3. \( \delta\) of N178\( \delta\) of N178 of 14-3-3 formed a hydrogen bond with the backbone amide nitrogen of F266\( \delta\) of F266 of Numb or L285\( \delta\) of L285 of Numb, respectively. N\( \delta\) of N229\( \delta\) of N229 from each monomeric 14-3-3 subunit formed a hydrogen bond with the backbone carbonyl oxygen of G264\( \delta\) of G264 of Numb or L283\( \delta\) of L283 of Numb, respectively. At the phospho-site, the phosphate was engaged in a positively charged pocket formed by residues R57 and R132 of 14-3-3, and a hydrogen bond formed between the phospho and the side chain of Y133\( \delta\) of Y133 of 14-3-3 further stabilized the interaction (Fig. 2D). Note that the above contacts between the phospho and 14-3-3 are completely conserved in the canonical mode 1 and mode 2 14-3-3/target interactions (34) (Fig. 2E). However, due to the lack of further contacts, e.g., the hydrogen bonds formed between S (-2) of the phospho-peptide and E180 and W228 of 14-3-3 observed in mode 1 interaction, or the salt bridges formed between R (-4) of the
phospho-peptide and E180 of 14-3-3 observed in mode 2 interaction (Fig. 2E), single phospho-Numb peptides (pS265 or pS284) could not bind to 14-3-3η robustly. In line with the structure, alanine mutations of R57 and/or R132 in 14-3-3η completely disrupted its interaction with Numb pSS (Fig. S1A). Together, the non-canonical Numb pSS peptide bound to the 14-3-3 η dimer in a bidentate fashion by occupying the canonical phospho-sites.

The DPF motif is not sufficient for Numb to form a stable complex with α-appendage

An interesting phenomenon is that the aPKC/CaM-KI-mediated phosphorylation of Numb (at S265 and S284) and the simultaneous recruitment of 14-3-3 dissociated the stable Numb/AP2 complex in vitro (28), and led to the diffusion of Numb from the AP2-marked CCPs in living cells (19). It seems that the interaction between Numb and α-appendage in the AP2 complex is independent of Numb phosphorylation (24,28), and thus, the Numb/AP2 dissociation may not be a direct consequence of Numb phosphorylation. In line with this hypothesis, Flag-tagged full-length Numb WT interacted with GST-α-appendage equally well when pre-incubated with or without phosphatase inhibitor (Fig. 3A), implying that the phosphorylation state of Numb does not affect its interaction with α-appendage. Compared with Numb WT, the S265A or S265,S284A mutation, which disrupted the binding of Numb to 14-3-3, had no observable effect on the binding to α-appendage (Fig. 3A). Whereas in the competition assay, the amount of Flag-Numb pulled down by GST-α-appendage was gradually reduced in the presence of increasing amounts of 14-3-3 (Fig. 3B), indicating that 14-3-3 directly competed with α-appendage for Numb binding.

As the well-known D\textsuperscript{556}PF motif responsible for α-appendage binding is located on the very C-terminal of Numb (Fig. 3C), it is not clear how 14-3-3 binding to phospho-Numb (around S265 and S284) would interfere with its interaction with α-appendage. An attractive hypothesis is that there is another important α-appendage binding site located near that region. We thus mapped the specific α-appendage binding site(s) within Numb by GST pull-down assay. We first confirmed that the C-terminal part of Numb (aa 241-593, referred to as NumbC hereafter) but not the PTB domain (aa 1-240) was responsible for binding to α-appendage (Fig. 3C and 3D). In line with our cell lysate GST pull-down results (Fig. 3A), the phosphorylation mimic S265E, S284E, or S265,S284E mutants of NumbC bound to α-appendage with a similar affinity as WT did (Fig. 3E). Note that the Numb fragment containing the C-terminal DPF motif alone (aa 541-593) had a very weak binding affinity to α-appendage (Fig. 3C and 3D), further implying the existence of other α-appendage-binding motif within Numb. Unfortunately, NumbC was very unstable, possibly due to its long unstructured random coil region with easy access for proteolysis. To find a relatively stable Numb fragment capable of binding to α-appendage as efficiently as NumbC, we made several truncation fragments of NumbC covering both the 14-3-3 binding region and the C-terminal DPF motif, and we finally found a satisfactory Numb fragment (aa 260-329 fused with aa 541-570, referred to as NumbF hereafter; Fig. S2). Note that though there are two DPF motifs (D\textsuperscript{333}PF and D\textsuperscript{556}PF) within Numb (Fig. 3C), D\textsuperscript{333}PF plays a minor role in binding to α-appendage (Figs. 3C and S2), which is consistent with previous reports (22,26).

Importantly, either the N-terminal part (aa 260-329) or the D\textsuperscript{556}PF motif showed very weak to moderate binding to α-appendage, and the D\textsuperscript{556}PF motif could not form a stable complex with α-appendage, as NumbF did, when analyzed by analytical gel-filtration assay (Fig. 3D and 3F). When α-appendage was incubated with both NumbF and the D\textsuperscript{556}PF motif in a 1:1:1 molar ratio, only NumbF was found in complex with α-appendage (Fig. 3F). Together, these results demonstrated that the D\textsuperscript{556}PF motif was essential but not sufficient for Numb binding to α-appendage, and the N- and C-terminal fragments of NumbF functioned cooperatively to interact with α-appendage with high affinity.

To understand how the two sites within NumbF collaboratively interact with α-appendage, we tried to obtain crystals of α-appendage in complex with NumbC, NumbF, or other Numb constructs. However, though we
could obtain a stoichiometric Numb/α-appendage complex (Figs. 3F and S2), our extensive attempts to grow crystals have failed.

**Another target binding site on α-appendage**

As the aPKC/CaM-KI phosphorylation-induced 14-3-3 recruitment of Numb impaired its binding to α-appendage, we were motivated by the idea that the other unknown α-appendage binding site might be partially overlapped with the 14-3-3 binding site in Numb, e.g., near the S265 and S284 phosphorylation sites. In line with this hypothesis, truncation of amino acids 260-290 (encompassing both phosphorylation sites) of full-length Numb dramatically weakened its binding ability to α-appendage (Fig. 4A). As a positive control, alanine substitution of the D556PF motif (referred to as DPF/A hereafter) robustly impaired the binding, and the combined mutation of Δ260-290 and DPF/A completely disrupted the interaction (Fig. 4A). We then constructed several N-terminal truncation fragments of NumbF and tested their binding to α-appendage. Truncation of the N-terminal 10 amino acids (Δ260-269) significantly weakened the binding 2-fold, whereas truncation of the N-terminal 20 amino acids (Δ260-279) resulted in a comparable though slightly weaker binding affinity to α-appendage than that of the Δ260-269 mutant (Fig. 4B), indicating that amino acids 260-269 of Numb may play an important role in α-appendage binding.

Sequence alignment of Numb revealed two evolutionarily conserved clusters within amino acids 260-269 of Numb: the bulky hydrophobic cluster (F266, F269) and the positively charged cluster (R262, Q263, R267; Fig. 4C). Alanine substitution of each cluster (referred to as FF/A and RQR/A respectively) had negligible influence on the Numb/α-appendage interaction, whereas the combined mutation of both clusters (referred to as RQFRF/A) significantly weakened the interaction to an extent comparable with that of the Δ260-269 mutant (Figs. 4B, 4D, S2A and S3A), indicating that both clusters act synergistically in α-appendage binding. As expected, the mutation of both RQFRF and D556PF motifs further weakened the Numb/α-appendage interaction, as indicated by the increased $K_d$ (Fig. 4D and 4E).

Next, we tried to map out the corresponding binding site of the RQFRF motif on α-appendage. Previous studies had identified the “top” and “side” target binding sites on α-appendage, which recognize the DPF/FxDxF/FxxFxxL and WVxF motifs, respectively (Fig. 4F) (7,9,40). Site-directed mutagenesis in α-appendage revealed that the top site (W840A) played an important role in binding Numb (most probably through the Δ556PF motif), whereas the side site (F740E) seemed not to be involved in the interaction (Figs. 4D, 4G and S2B), implying that other site distinct from these two sites may exist on α-appendage for accommodating the RQFRF motif. As both the hydrophobic and positive-charge properties of the RQFRF motif were required for its interaction with α-appendage (Fig. S3A), the corresponding properties should be present on the binding site of α-appendage. Surface analysis of α-appendage revealed two potential RQFRF-binding sites that are hydrophobic patches surrounded by negatively charged amino acids: the “E” cluster (containing E830, E863, E878 and E879) and the “ED” cluster (containing E702, D703, D760, D761, E797, E932, and E936; Fig. S3B). Mutation in the “E” cluster (by substituting the E residues with alanine, referred to as E/A) weakened the binding to NumbF by 2-fold, whereas mutation in the “ED” cluster (by substituting the E/D residues with alanine, referred to as ED/A) had an undetectable influence on the interaction (Fig. 4D and 4G). Thus, it is attractive for us to propose that the central RQFRF motif and the C-terminal DPF motif of Numb bind to the “E” cluster and “top” site of α-appendage, respectively, to cooperatively interact with α-appendage with high efficiency.

**Discussion**

It is well-documented that the important endocytic protein Numb is incorporated into the clathrin-coated pits/vesicles via the recognition of its C-terminal D556PF motif by the α-appendage of AP2, where it acts as an adaptor to further recruit cargos into CCPs/CCVs for trafficking (22,24,26). Intriguingly, the aPKC/CaM-KI-mediated phosphorylation of Numb at S265 and S284 results in the dissociation of Numb from AP2, which coincides with the...
recruitment of 14-3-3 by phospho-Numb (25). In this study, we discovered a conserved RQFRF motif located adjacent to the phosphorylation sites (S265 and S284) of Numb, which cooperated with the DPF motif to bind to α-appendage with high affinity. Upon phosphorylation at S265 and S284, Numb recruits 14-3-3, leading to the release of the adjacent RQFRF motif from α-appendage through steric hindrance, and then the DPF motif of Numb dissociates from the “top” site on α-appendage due to instability (Fig. 5). This finding defines the RQFRF motif of Numb as a triggering site necessary for the dynamic dissociation of the Numb/AP2 complex.

As the key component of the AP2 complex, α-appendage acts by recruiting a large number of accessory/regulatory proteins into CCPs/CCVs (6-8). During the past decades, two conserved target binding sites have been identified on α-appendage, namely, the “top” site that recognizes the DPF/FxDF/FxxFxL motifs and the “side” site that binds to the WVx/F/W motif (7,9,40). The “E” cluster discovered here, which could accommodate the RQFRF motif of Numb, provides a new mode for target recognition of α-appendage. Multiple sequence alignment showed that both the “E” cluster and the hydrophobic amino acids around it are evolutionarily highly conserved (Fig. S4), implying that the identified “E” cluster may be another general target binding site on α-appendage, and new targets of α-appendage could be explored. Importantly, the “E” cluster could be regarded as a gatekeeper site, necessary for robust binding to certain targets (e.g., Numb) but with almost no intrinsic affinity to the target itself, which therefore allows modulated target binding at this site by competitive regulatory factors (e.g., Numb). This dual-site recognition mode seen in Numb might be common in other α-appendage cargos, allowing the spatiotemporal fine-tuning of cargo recruitment and the release of the AP2 complex.

The structure of the Numb pSS/14-3-3η complex solved here reveals the high diversity of 14-3-3 target recognition. It has been shown that a synthetic peptide containing two chemically linked identical 14-3-3 sites gained a 30-fold higher affinity for 14-3-3 over a single 14-3-3 site (34). Meanwhile, the cooperation of a divergent 14-3-3 site (with extremely weak affinity to 14-3-3) and a consensus 14-3-3 site (with a modest affinity to 14-3-3) could also achieve markedly enhanced target binding (39,41). In the extreme Numb/14-3-3 case, neither of the phospho-sites in Numb was the canonical 14-3-3 site, and the mono-phospho-peptide had barely detectable binding to 14-3-3η. Nevertheless, both diverse 14-3-3 sites function cooperatively to interact with 14-3-3 with high efficiency. Our finding expands the potential target pool of 14-3-3.

Materials and methods

**Protein expression and purification**

Various mouse Numb fragments (Fig. 3), mouse α-adaptin appendage domain (aa 693-938), and mouse 14-3-3 (aa 1-246) were individually cloned into pGEX-6P-1 or a modified version of the pET32a vector (42), with the resulting protein containing a GST or Trx-His tag in its N terminus. All of the mutations were created by a standard PCR-based mutagenesis method and confirmed by DNA sequencing. Recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) host cells at 16 °C and were purified by using GST or Ni²⁺-NTA agarose affinity chromatography followed by size-exclusion chromatography.

**GST pull-down assay**

For the GST pull-down assay, various GST-tagged α-appendage proteins (4 nmol) were first loaded onto GSH-Sepharose 4B slurry beads and then incubated with 12 nmol potential binding partner proteins in 500 µL of assay buffer [containing 50 mM Tris (pH 8.0), 100 mM NaCl, 1 mM DTT, and 1 mM EDTA] at 4 °C for 1 h. After being washed three times, proteins captured by the affinity beads were eluted by boiling, resolved by 12% SDS-PAGE, and detected by Coomassie blue staining.

**Cell lysate GST pull-down assay**

Human HEK293T cells were transiently transfected with 6 µg of full-length Flag-Numb WT or various mutants using polyethylenimine transfection reagent. Cells were collected 24 h post-transfection and lysed in a buffer containing 50 mM Tris (pH 7.4), 150 mM sodium chlorate, 0.5% Nonidet P-40, 10 mM sodium fluoride, 1
mM sodium metavanadate, 1 mM DTT, and 10 mM PMSF with or without phosphatase inhibitors. Each lysate was incubated with GST-fusion proteins at 4 °C for 2 h. For the competition assay, the lysate was incubated with GST-α-appendage protein in the presence or absence of 0.1 mg, 0.4 mg or 0.8 mg purified His-14-3-3.

**In vitro kinase assay**

Approximately 1 mg/mL purified Trx-tagged WT or mutant mouse NumbC (aa 241-593) was dialyzed in the kinase buffer containing 20 mM MOPS (pH 7.4), 15 mM MgCl$_2$, and 2 mM DTT. GFP-tagged PKC$\iota$ was transfected in HEK293T cells and harvested 24 h after transfection. The cell lysate was incubated with GFP antibody-coupled Protein G beads. After being washed with the lysis buffer and the kinase assay buffer, respectively, PKC$\iota$-bound beads were mixed with 50 µL of NumbC sample, and incubated at 25 °C for 1.5 h in the kinase assay buffer containing 0.1 mM ATP.

**Immunoblotting**

After extensive washing of the beads with the lysis buffer, the captured proteins were boiled in SDS-PAGE loading buffer and subjected to SDS-PAGE. Proteins were transferred to a 0.45 mM nitrocellulose membrane (Millipore), and the nitrocellulose membrane was blocked with 3% BSA in TBST [20 mM Tris-HCl (pH 7.4), 137 mM NaCl and 0.1% Tween-20] buffer at room temperature for 1 h, followed by incubation with the anti-GFP (ABclonal, AE012), anti-Flag (ABclonal, AE005) or anti-His (Abcl, ABT505) antibody at a 1/3,000 dilution at 4 °C overnight. Membranes were washed three times with TBST buffer, incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (ABclonal, AS003) and visualized on a LAS3000 chemiluminescent imaging system.

**Analytical gel filtration assay**

Analytical gel filtration experiments were carried out on an AKTA FPLC system (GE Healthcare). Proteins (20 µM, 100 µL) were loaded on a Superose 12 10/300 GL column 20 (GE Healthcare) equilibrated with buffer containing 50 mM Tris (pH 8.0), 100 mM NaCl, 1 mM DTT and 1 mM EDTA. Protein elution was detected by the absorbance at 280 nm.

**Crystallography**

Freshly purified 14-3-3 was concentrated to 40 mg/mL, and crystals were grown by the hanging drop vapor diffusion method at 16 °C in a reservoir solution containing 100 mM tri-sodium citrate dehydrate (pH 5.6), 20% isopropanol, and 20% polyethylene glycol 4000. The diffraction data of the crystals were collected at the SSRF (Shanghai Synchrotron Radiation Facility in China) beamline BL17U1 at a wavelength of 0.9792 Å. The data were processed and scaled using HKL2000. The phasing problem of 14-3-3 and Numb peptide was solved by molecular replacement using the 14-3-3 structure (PDB ID: 4HKC) as the search model against the 2.1 Å resolution dataset. The initial model was further rebuilt, adjusted manually with COOT (43) and refined by the phenix.refine program of PHENIX (44). The final model had 98.4% of the residues in the favored region of the Ramachandran plot with no outliers. The final refinement statistics are summarized in Table 1.

**ITC measurements**

ITC measurements were performed on an ITC200 Micro calorimeter (MicroCal) at 25 °C. All of the protein samples were dissolved in a buffer containing 50 mM Tris (pH 8.0), 1 mM EDTA, and 100 mM NaCl. The titrations were carried out by injecting 40-µL aliquots of various Numb fragments (0.5–0.7 mM) or phosphorylated Numb peptides into solutions of α-appendage or 14-3-3 (0.03–0.05 mM) at time intervals of 2 min to ensure that the titration peak returned to the baseline. The titration data were analyzed using the program Origin 7.0 and fitted by the one-site binding model.
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Author Contributions: X.C. and W.W. conceived the research and analyzed data. X.C., W.Y., and A.G. performed the biochemical experiments. Z.L. and Z.S. solved the crystal structure. X.C. and W.W. wrote the manuscript. W.W. coordinated the research.

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**FOOTNOTES**

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The coordinates of 14-3-3 and Numb peptide have been deposited in the PDB with accession code 5YQG.

The abbreviations used are: aa, amino acids; GST, glutathione S-transferase; DTT, DL-Dithiothreitol; CaM-KI, Ca^{2+}/calmodulin-dependent protein kinase I; aPKC, atypical protein kinase C; pSS, pS265S284; ITC, isothermal titration calorimetry; NumbC, C-terminal part of Numb, aa 241-593; NumbF, Numb fusion fragment, aa 260-329 fused with aa 541-570; WT, wild type; SSRF, Shanghai Synchrotron Radiation Facility in China; EDTA, ethylene diamine tetraacetic acid; PCR, polymerase chain reaction; Ni^{2+}-NTA, nickel-nitrilotriacetic acid; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethlysulfonyl fluoride; BSA, Bovine Serum Abumin.
Table 1. Data collection and refinement statistics

|                          | Numb pSS/14-3-3 complex |
|--------------------------|-------------------------|
| **Data collection**      |                         |
| Space group              | *P1211*                 |
| Cell dimensions          |                         |
| $a, b, c$ (Å)            | 59.260, 74.626, 134.136 |
| $\alpha, \beta, \gamma$ (°) | 90.000, 90.000, 90.000 |
| Wavelength (Å)           | 0.9792                  |
| Resolution (Å)           | 50.00-2.10 (2.14-2.10)* |
| $R_{\text{merge}}$ (%)   | 8.6 (76.6)              |
| Mean $I/I$               | 16.5 (2.1)              |
| Completeness (%)         | 98.2 (100)              |
| Redundancy               | 3.5 (3.7)               |
| **Refinement**           |                         |
| Resolution (Å)           | 49.90-2.10              |
| No. reflections          | 66, 596                 |
| $R_{\text{work}} / R_{\text{free}}$ | 18.2 / 22.2          |
| No. atoms                |                         |
| Protein                  | 7, 379                  |
| Water                    | 490                     |
| B factors                | 34.70                   |
| R.m.s deviations         |                         |
| Bond lengths (Å)         | 0.007                   |
| Bond angles (°)          | 0.819                   |
Figure Legends

Figure 1. Phosphorylation of Numb at S265 and S284 recruits 14-3-3. A, Amino acid sequences of the Numb phospho-peptides. The S265 and S284 phosphorylation sites are indicated. B, ITC-based measurements of the binding between Numb phospho-peptides and 14-3-3η. C, Cell lysate GST pull-down assay showing that full-length wild-type (WT) Numb but not the S265A, S284A, or S265,284A mutants could be pulled down by GST-14-3-3. D, Both 14-3-3 sites in Numb are required for stable interaction with 14-3-3. In this experiment, Trx-His-tagged Numb 241-593 WT or mutants were pretreated with affinity purified GFP-PKCι and then mixed with GST-14-3-3 (see ‘Materials and methods’ for details). The Numb 241-593 WT or mutant proteins were detected by western blot using anti-His antibody.

Figure 2. Crystal structure of 14-3-3η in complex with di-phosphorylated Numb pSS peptide. A, Ribbon diagram representation of the Numb pSS (orange)/14-3-3 (blue and green) complex as viewed from the side. The phosphate group is drawn as a ball-and-stick model. B, Combined ribbon and surface diagram of the complex as viewed from the top. C, The 2Fo-Fc map for the Numb pSS peptide contoured at 1.0 σ. D, Polar interactions (dotted lines) between the Numb pSS peptide and 14-3-3. E, Polar interactions between 14-3-3 and the canonical mode 1 and mode 2 motifs.

Figure 3. Discovery of another α-appendage binding site within Numb. A, The interaction between Numb and α-appendage was phosphorylation independent. HEK293T cells were transfected with full-length Flag-Numb WT or various mutants. Lysates were incubated with GST-α-appendage with or without phosphatase inhibitor. B, Competition assay. 14-3-3 captured phosphorylated Numb from α-appendage in a dose-dependent manner. The amounts of 14-3-3 were 0.1 mg, 0.4 mg and 0.8 mg. Lysates were pre-incubated with phosphatase inhibitor. C, Mapping the α-appendage binding region of Numb. A summary of the binding affinities between various Numb constructs and α-appendage, as shown in panel D, is given on the right. D, GST pull-down experiments showing that NumbC (aa 241-593) and NumbF (aa 260-329+541-570) bound to α-appendage with high affinity, whereas the C-terminal DPF motif (aa 541-593) showed weak binding to α-appendage. E, Phosphorylation mimic S/E mutations on NumbC had undetectable impact on Numb/α-appendage interaction. F, An analytical gel filtration assay showed that NumbF but not the C-terminal DPF motif (aa 541-593) could form a stable complex with α-appendage. The elution volumes of the molecular size markers are indicated by arrowheads at the top.

Figure 4. Mapping the other Numb/α-appendage binding site. A, Cell lysate GST pull-down assay showing that amino acids 260-290 of Numb played an important role in binding to α-appendage. B, Representative SDS-PAGE analysis and quantification data showing that N-terminal truncation of NumbF significantly decreased its binding affinity to α-appendage. C, Multiple sequence alignment of mouse (mNumb), rat (rNumb), human (hNumb), bison (bNumb), nanorana (nNumb), zebrafish (zNumb), and Drosophila (dNumb) Numb color-coded by sequence identity in green. The residues responsible for α-appendage binding are indicated with a red circle. D, Summary of the ITC-based measurement of the binding affinities between NumbF WT or various mutants and α-appendage proteins. E, Both RQFRF (the bulky hydrophobic cluster F266, F269 and the positively charged cluster R262, Q263, R267) and the C-terminal DPF motifs contributes to efficient α-appendage binding. F, Surface diagram of α-appendage, with the “top” site, “side” site and “E” cluster indicated. G, Mapping the Numb binding sites on α-appendage by point mutation. E/A indicates E830,E863,E878,E879A, and ED/A indicates E702,D703,D760,D761,E932,E936A. All statistical data in this figure represent the results of three independent batches of experiments and are expressed as the mean ± SD.

Figure 5. Model of the 14-3-3-regulated dissociation of the Numb/AP2 complex during the endocytic process. The central RQFRF motif and the C-terminal DPF motif of Numb cooperatively interact with
α-appendage with high efficiency, by binding to the “E” cluster and the “top” site, respectively. The aPKC/CaM-KI-mediated phosphorylation of Numb at S265 and S284 recruits 14-3-3 and disrupts the binding between the RQFRF motif and the “E” cluster. The interaction between the DPF motif and the “top” site is not sufficient to maintain Numb/α-appendage as a stable complex, and Numb then dissociates from AP2. For clarity, β2-appendage is omitted in the figure.
Structural basis of Numb binding to α-adaptin and 14-3-3

Figure 1

A

B

C

D

Numb

pS265

pS284

pSS

260

265

284

289

LARGPpSFRGFpASQKMSPFKRQLSRLN

Flag-Numb

IB: Flag

GST-14-3-3

Lysate

IB: Flag

GST Pull-down

WT

50 μM

5 μM

Marker (Da)

IB: His

GST 14-3-3

Lysate PKC

Irx-His-Numb

241-593

Time (min)

0

10

20

30

40

Numb pS265

14-3-3 dimer

kcal/mol/n molec

0.0

1.0

2.0

3.0

4.0

Molar Ratio

Numb pS284

14-3-3 dimer

kcal/mol/n molec

0.0

1.0

2.0

3.0

4.0

Molar Ratio

Numb pSS

14-3-3 dimer

kcal/mol/n molec

0.0

1.0

2.0

3.0

4.0

Molar Ratio

Kd = 0.32 ± 0.02 μM

N= 1
Figure 2

Structural basis of Numb binding to α-adaptin and 14-3-3
Structural basis of Numb binding to α-adaptin and 14-3-3

Figure 3
Figure 4

A) GST Pull-down

B) GST-α-appendage

C) mNumb = ARQGFRGFPALSQKMBPFKRCQLRINE

D) α-appendage

E) Numb WT

F) "Top" site "WxxF" cluster

G) Numb 260-329+541-570

H) Numb WT

I) GST-α-appendage WT

J) GST-α-appendage WT

K) GST-α-appendage WT

L) GST-α-appendage WT

M) GST-α-appendage WT

N) GST-α-appendage WT

O) GST-α-appendage WT

P) GST-α-appendage WT

Q) GST-α-appendage WT

R) GST-α-appendage WT

S) GST-α-appendage WT

T) GST-α-appendage WT

U) GST-α-appendage WT

V) GST-α-appendage WT

W) GST-α-appendage WT

X) GST-α-appendage WT

Y) GST-α-appendage WT

Z) GST-α-appendage WT

| α-appendage | Kd (μM) |
|------------|---------|
| WT         | 12.0 ± 0.9 |
| Δ260-279   | 55.8 ± 3.8 |
| DPF/A      | >100     |
| RQRF/A     | 47.8 ± 6.3 |
| RQRF/A,DPF/A | >300    |

NumbF

WT         12.0 ± 0.9
WR46A      N/A
F740E      11.3 ± 0.8
E/A        48.6 ± 5.3
ED/A       16.9 ± 1.3
Structural basis of Numb binding to α-adaptin and 14-3-3

Figure 5
Structural determinants controlling 14-3-3 recruitment to the endocytic adaptor
Numb and dissociation of the Numb/α-adaptin complex
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