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# The NECAP PHear domain increases clathrin accessory protein binding potential

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**AP-2 is a key regulator of the endocytic protein machinery driving clathrin-coated vesicle (CCV) formation. One critical function, mediated primarily by the AP-2 α-ear, is the recruitment of accessory proteins. NECAPs are α-ear-binding proteins that enrich on CCVs. Here, we have solved the structure of the conserved N-terminal region of NECAP 1, revealing a unique module in the pleckstrin homology (PH) domain superfamily, which we named the PHear domain. The PHear domain binds accessory proteins bearing FxDxF motifs, which were previously thought to bind exclusively to the AP-2 α-ear. Structural analysis of the PHear domain reveals the molecular surface for FxDxF motif binding, which was confirmed by site-directed mutagenesis. The reciprocal analysis of the FxDxF motif in amphiphysin I identified distinct binding requirements for binding to the α-ear and PHear domain. We show that NECAP knockdown compromises transferrin uptake and establish a functional role for NECAPs in clathrin-mediated endocytosis. Our data uncover a striking convergence of two evolutionarily and structurally distinct modules to recognize a common peptide motif and promote efficient endocytosis.**

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## Introduction

The formation of endocytic clathrin-coated vesicles (CCVs) is a complex multistep process requiring coordination of a low-affinity interaction network based on protein–protein and protein–lipid interplay (Ritter and McPherson, 2004; Traub, 2005). These interactions are mediated through protein modules that specifically recognize lipid head groups and/or peptide motifs in target molecules (McPherson and Ritter, 2005; Traub, 2005). One hub for the organization of this machinery is the α-ear of the heterotetrameric clathrin adaptor AP-2 (Owen *et al*, 2004; Honing *et al*, 2005). The α-ear, which is connected to the AP-2 trunk by a flexible linker, extends into the surrounding cytosol to recruit endocytic accessory proteins through N-terminal platform and C-terminal sandwich subdomains that each present a conserved protein-binding interface. Through these interactions, the α-ear functions as a major organizer for numerous endocytic accessory proteins including Eps15, epsins, amphiphysin I and II, AP180, connecdenn, NECAP 1 and 2, synaptojanin, and AAK1 (McPherson and Ritter, 2005; Allaire *et al*, 2006). The platform subdomain utilizes overlapping binding sites to interact with DPF/W, FxDxF, and FxxFxxL peptide motifs (Owen *et al*, 1999; Traub *et al*, 1999; Brett *et al*, 2002; Praefcke *et al*, 2004). Recently, we and others identified a spatially distinct binding site on the sandwich subdomain that recruits proteins through WxxF-acidic motifs (Ritter *et al*, 2003; Jha *et al*, 2004; Mishra *et al*, 2004; Praefcke *et al*, 2004; Ritter and McPherson, 2004; Walther *et al*, 2004).

The NECAPs are a family of endocytic proteins that we identified through a proteomic analysis of CCVs isolated from rat brain (Wasiak *et al*, 2002; Ritter *et al*, 2003; Blondeau *et al*, 2004). NECAP 1 expression levels are highest in the brain and dominant-negative NECAP 1 constructs disrupt synaptic vesicle endocytosis (Murshid *et al*, 2006). NECAP 1 is also expressed in non-neuronal tissues and cells and NECAP 2 is ubiquitously expressed. In contrast to most endocytic accessory proteins, both NECAP family members are enriched on CCVs together with AP-2 and clathrin (Mills *et al*, 2003; Ritter *et al*, 2003; Blondeau *et al*, 2004). The primary NECAP sequences are evolutionarily conserved but lack homology to other proteins. Our initial studies identified and characterized C-terminal peptide-binding motifs for AP-2 and the Golgi/endosome-associated clathrin adaptor proteins AP-1/GGAs (Ritter *et al*, 2003, 2004; Mattera *et al*, 2004).

Here, we have solved the structure of the conserved N-terminal region in NECAP 1, revealing a new module in the pleckstrin homology (PH) domain superfamily. The PH domain superfamily is one of the largest domain superfamilies and includes in addition to PH domains, phosphotyrosine-binding (PTB), Ena/VASP homology 1 (EVH1)/WASP homology 1 (WH1), and Ran-binding domains (RanBD) (Blomberg *et al*, 1999). The PH superfold is a seven-stranded β-barrel that is closed on one side by a C-terminal α-helix. PH domains were first characterized as phospholipid-binding modules, but further studies revealed that only a few have high affinity for phospholipids (Lemmon, 2004; DiNitto and Lambright, 2006). In budding yeast, for example, only one of 33 PH domains in the genome strongly binds phospholipids (Yu *et al*, 2004). Many PH domains have now been

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recognized to bind proteins (Lemmon, 2004). Besides the common fold, the superfamily is characterized by low sequence similarity between family members and high variability in the length of the loops connecting the core structural elements (DiNitto and Lambright, 2006). This flexibility allows the stable PH superfold to morph into interfaces with the electrostatic potential and molecular surface necessary to adopt variable functions. Characterization of the PH-like domain of NECAPs identified this module as a protein-binding interface that mimics the FxDxF motif binding properties of the α-ear. We have thus named the module the PHear (PH fold with ear-like function) domain. As NECAPs themselves are stable components of CCVs, the PHear domain provides a means to regulate the access of FxDxF motif-bearing proteins to the assembling clathrin coat, thereby fine-tuning clathrin-mediated endocytosis.

## Results

### *The PHear fold*

The N-terminal half of NECAPs is well conserved throughout evolution (Figure 1A and Supplementary Table I). Analysis of $^{15}$N-$^{1}$H HSQC spectra of an $^{15}$N-enriched NECAP 1 construct encoding amino acids 1–178 revealed good signal dispersion for amino acids 1–132, while HSQC signals for amino acids 134–178 were located between 7.8–8.3 p.p.m. for $^{1}$H nuclear magnetic resonance (NMR) and their heteronuclear $^{15}$N{$^{1}$H}-NOEs were negative (data not shown), indicating that only residues 1–132 are folded. We thus focused on a shorter NECAP 1 construct encoding amino acids 1–133, which showed good signal dispersion (5A).

The fold of amino acids 1–133 was determined using triple-resonance, multidimensional NMR spectroscopy on $^{15}$N- and $^{13}$C-,$^{15}$N-labeled samples. The structural statistics of the analysis are given in Table I and a ribbon presentation and backbone superposition for the deposited structure is shown in Figure 1B and C. The NECAP PHear domain possesses a β-barrel fold, with seven β-strands packed together to form an antiparallel β-sandwich, closed by a long C-terminal α-helix (Figure 1B and C). A second, short helical structure is formed by amino acids 33–35 in the loop between β-strands 1 and 2 (Figure 1B and C). The hydrophobic core of the fold is formed primarily by residues V17, V19, L47, I49, L58, I60, V74, V82, I94, I96, and I106. A comparison of the C$^{\alpha}$-atomic coordinates of the NECAP 1 PHear domain to known protein structures using the DALI program revealed 31 similar structures in the PDB database, with a *Z*-factor in the range of 5.0 to 6.6 (a total number of 84–110 equivalent residues; Supplementary Table II), indicating a high statistical significance for these structural domain alignments. The selected structures all share the common PH superfold (Blomberg *et al*, 1999). Within the PH domain superfamily, the NECAP fold best resembles PH domains.

PH domains were initially characterized as phospholipid-binding modules. NMR titrations of the PHear domain with sodium phosphate and inositol-1,4,5-tris-phosphate revealed

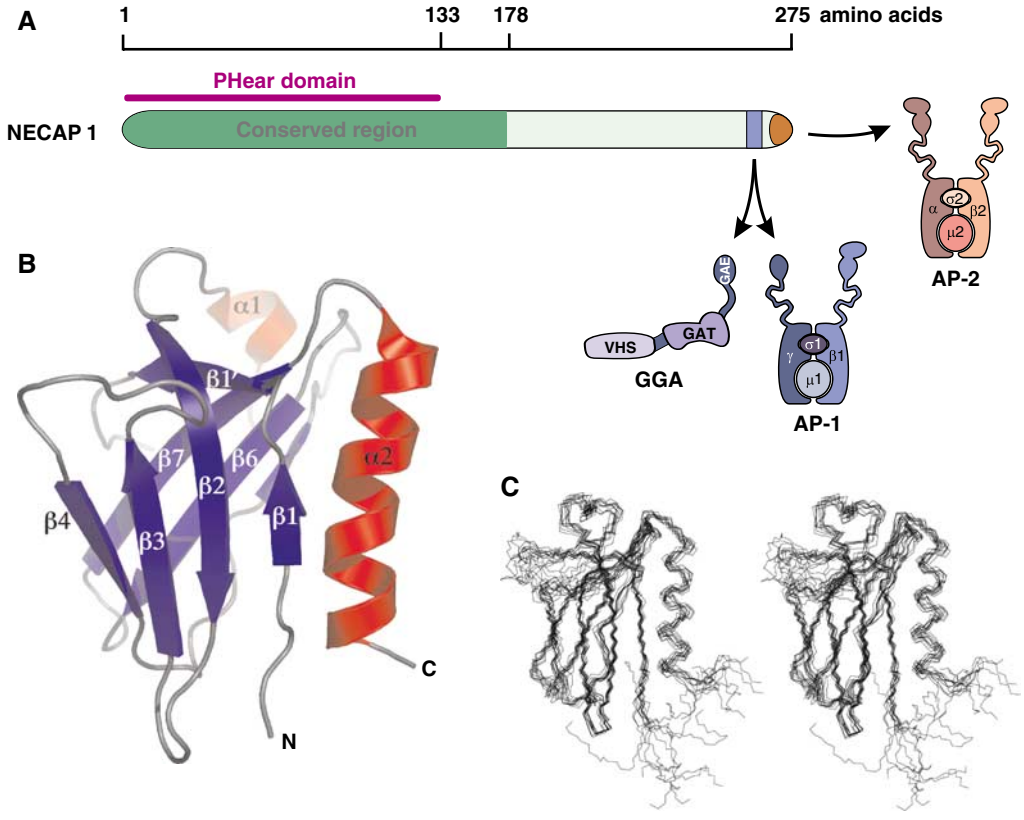



**Figure 1** The NECAP fold. (**A**) Schematic representation of the organization of NECAP 1. The evolutionarily conserved N-terminal region (amino acids 1–178) is represented in green and the portion encoding the PHear fold (amino acids 1–133) is indicated by the purple line. The C-terminal binding motifs for AP-1/GGAs (blue) and AP-2 (brown) are assigned to the corresponding adaptor protein. (**B**) Ribbon representation of the solution structure of the NECAP 1 PHear domain with β-strands and α-helices indicated. (**C**) Stereoview of the backbone superposition of 10 low-energy structures.

**Table I** Structural statistics for the NECAP 1 PHear domain

| | |
|---|---|
| *Restraints for structure calculations* | |
| Intra-residue NOEs | 258 |
| Sequential NOEs | 291 |
| Medium- and long-range NOEs | 148 |
| Hydrogen bonds | 42 |
| $\phi$ Backbone angles | 118 |
| NH residual dipolar couplings | 110 |
| *Final energies (kcal/mol)* | |
| $E_{total}$ | 329±20 |
| $E_{bond}$ | 13.3±1.6 |
| $E_{angle}$ | 83±7 |
| $E_{impr}$ | 9.4±1.4 |
| $E_{repel}$ | 152±23 |
| $E_{noe}$ | 10.6±3.1 |
| $E_{cdih}$ | 0.4±0.3 |
| $E_{sani}$ | 30±6 |
| $E_{coup}$ | 31±6 |
| *R.m.s.d. from idealized geometry* | |
| Bond (Å) | 0.0025±0.0002 |
| Bond angles (deg.) | 0.38±0.02 |
| Improper torsions (deg.) | 0.23±0.02 |
| *R.m.s.d. for experimental restraints* | |
| Distances (Å) | 0.014±0.002 |
| Dihedral angles (deg.) | 0.12±0.08 |
| *Residual dipolar couplings* | |
| R.m.s.d. (Hz) | 2.4±0.2 |
| *Q*-value | 0.16±0.02 |
| *Coordinate r.m.s.d. from average structure (Å)*[a] | |
| Backbone atoms (N, $C^{\alpha}$, C′) | 0.70±0.07 |
| All heavy atoms | 1.37±0.07 |
| *Ramachandran analysis (%)* | |
| Residues in most favored regions | 66.1±4.4 |
| Residues in additional allowed regions | 26.1±6.1 |
| Residues in generously allowed regions | 5.6±2.2 |

[a]For residues 10–22, 32–61, and 69–129.

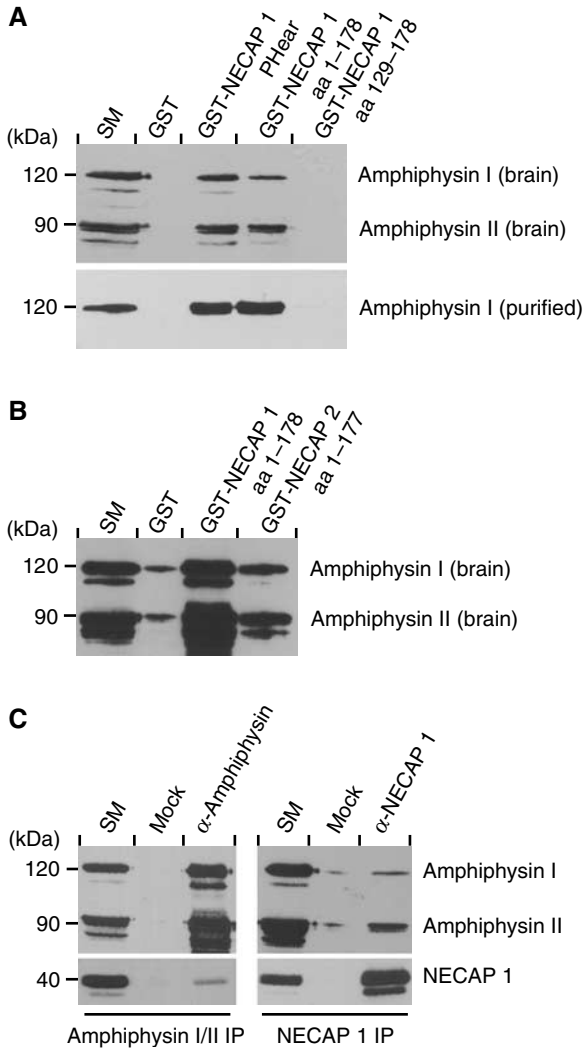



**Figure 2** NECAPs interact with amphiphysins. (**A**, **B**) GST fusion proteins encoding the NECAP 1 PHear domain, NECAP 1 aa 1–178, NECAP 1 aa 129–178, NECAP 2 aa 1–177, or GST alone pre-coupled to glutathione–Sepharose were incubated with Triton X-100-solubilized rat brain extract or purified amphiphysin I as indicated, and amphiphysin binding was analyzed by Western blot. (**C**) Co-immunoprecipitation of endogenous NECAP 1 and amphiphysins from Triton X-100-solubilized rat brain extract using antibodies directed against amphiphysin (1874, which recognizes both amphiphysin I and II) or NECAP 1. Immunoprecipitated proteins were detected by Western blot. For all binding studies, 10% of the protein amount used in each reaction is analyzed as starting material (SM).

only low-affinity interactions ($K_d \sim 10$ mM) for residues in the spacial proximity of R90 and R113, which are outside the signature phospholipid binding motif (Lemmon and Ferguson, 2001) (data not shown). Structural alignment of the NECAP PHear domain with phospholipid-binding PH domains shows that most of the critical amino acids for phospholipid interaction are not conserved (Supplementary Figure 1A). Moreover, we failed to detect phospholipid binding using PIP strips, despite binding of the epsin 1 ENTH domain (Supplementary Figure 1B), and lipid sedimentation assays failed to detect interaction of the PHear domain with purified brain lipids (Supplementary Figure 1C). We did, however, detect a weak interaction with brain lipids supplemented with 20% PtdIns(4,5)$P_2$. Further studies will be needed to determine the biological significance of this interaction, but taken together, these studies indicate that phospholipids are unlikely the primary target of the NECAP PHear domain.

### *Interaction with amphiphysin I*

To search for protein-binding partners in an unbiased screen, we performed affinity selection experiments from rat brain extracts with purified GST-NECAP 1 PHear domain and MS/MS analysis identified amphiphysin I as an interaction partner. Amphiphysin I is involved in clathrin-mediated endocytosis and forms homodimers as well as heterodimers with amphiphysin II (Butler *et al*, 1997; Ramjaun *et al*, 1997, 1999; Wigge *et al*, 1997; Di Paolo *et al*, 2002). Both isoforms contain an N-terminal BAR domain that senses membrane curvature and targets the protein to the neck of a forming vesicle (Peter *et al*, 2004), and a C-terminal SH3 domain that recruits synaptojanin and dynamin during vesicle formation (Ramjaun *et al*, 1997; McPherson, 1999). The interaction of the PHear domain with amphiphysin was readily detected by Western blot (Figure 2A). In pull-down assays from brain extract, amphiphysin binding is similar for the PHear domain and the longer NECAP 1 construct encoding amino acids 1–178, while the region of amino acids 129–178, which comprise the evolutionarily conserved region in NECAP 1 outside

the PHear fold (Figure 1A), does not contribute to amphiphysin binding (Figure 2A). Similar binding behavior was observed using purified GST-amphiphysin I, from which the GST tag had been removed before the binding assay, demonstrating that the NECAP PHear domain directly interacts with amphiphysin I (Figure 2A). Moreover, amphiphysin binding is conserved between NECAP 1 and NECAP 2 (Figure 2B). The NECAP 1/amphiphysin interaction was verified by co-immunoprecipitation of endogenous proteins in both directions (Figure 2C).

### *The PHear domain targets FxDxF motifs*

To identify the region in amphiphysin I responsible for PHear domain binding, we generated a series of GST-amphiphysin I C- and N-terminal deletion constructs (Figure 3A), which were used in pull-down assays from rat brain extract. NECAP 1 binding was observed for all constructs containing the amphiphysin insert domain, similar to what was seen for clathrin (Figure 3B). The insert domain is present in brain-specific splice variants of amphiphysins and contains in addition to clathrin-binding motifs (LLDLD and PWDLW), peptide motifs for interaction with SH3 domain proteins (PxxP) and the α-ear of AP-2 (FxDxF and DPF; Figure 3C) (McMahon *et al*, 1997; Micheva *et al*, 1997; Ramjaun and McPherson, 1998; Slepnev *et al*, 2000; Miele *et al*, 2004). We used a series of N- and C-terminal deletion constructs of the insert domain (Figure 3C) to define the PHear domain-binding region. The constructs showed the expected binding pattern for clathrin, as constructs containing a single clathrin-binding motif showed weak interaction while constructs containing both motifs showed strong clathrin binding (Figure 3D). AP-2 interaction was detected for all constructs containing the FxDxF motif, either alone or together with the single DPF motif (Figure 3D). AP-2 binding was not observed for constructs containing the DPF motif alone, due to the low affinity of a single DPF motif. Surprisingly, NECAP 1 demonstrated the same binding pattern as AP-2 (Figure 3D). In particular, both proteins bound to amphiphysin I constructs encoding amino acids 291–329 and 324–445, and the small overlapping region of residues 324–329 encodes the FxDxF motif known to target the α-ear of AP-2 (Brett *et al*, 2002). To verify that this region mediates the interaction with NECAP 1, we used a synthetic peptide containing the amphiphysin I FxDxF motif (CSF**FE**D**N**F**P**E) in competition binding studies and indeed, the synthetic FxDxF peptide interferes with amphiphysin binding to the NECAP 1 PHear domain (Figure 3E).

We used 2D-NMR spectroscopy to further characterize the PHear domain-binding motif in amphiphysin I. Significant chemical shift changes ($>0.1$ p.p.m.) in the $^{1}H$-$^{15}N$ HSQC spectra of an $^{15}N$-labeled amphiphysin I construct encoding amino acids 291–329 were found for residues 321–328 (ISF**FE**D**N**F; Figure 4A and B). Additional signal broadening for residues S322 to F328 suggests their involvement in ligand binding. To further investigate the contribution of single amino acids, we performed an alanine screen for positions 320–328 within the context of amino acids 311–329 and tested for binding of purified His-tagged α-ear and NECAP 1 to the various amphiphysin I mutants. This screen revealed interesting differences in the binding properties of the α-ear and NECAP 1. For the FxDxF motif core, binding of both proteins was affected by mutation of F324 and F328, whereas mutation of E325 and N327 did not inhibit binding (Figure 4C). A major difference in binding behavior is observed when changing the center core position D326 to alanine. This mutation strongly reduces binding to the α-ear but does not affect NECAP 1 binding (Figure 4C). D326 has been shown to directly contact the α-ear (Brett *et al*, 2002), but the extended definition of the motif, Fx[D/N/S]x[F/L] (Mishra *et al*, 2001), indicates a higher variability allowed for this position. The lack of influence of the D326A mutation on NECAP 1 binding reveals differences in binding requirements for the two domains, suggesting a higher tolerance of the PHear domain for variation of the middle position of the motif core. Our NMR study also detected significant chemical shift changes for positions preceding the motif core and for S322, concurrent signal broadening further suggests a direct contribution to binding. Mutation of this position to alanine selectively interferes with NECAP 1 binding and does not affect the interaction with the α-ear, whereas changing I321 to alanine does not influence interaction with either domain. Together, these data demonstrate that while both the α-ear and PHear domain use the FxDxF motif as targeting sequence, some positions within and surrounding the core contribute to affinity and binding specificity in a domain-specific manner.

### *The PHear domain-binding site*

We used NMR to identify the binding site for FxDxF motifs on the PHear domain. Chemical shift changes in the $^{1}H$-$^{15}N$ HSQC spectra of the $^{15}N$-labeled NECAP 1 PHear domain upon interaction with the amphiphysin I insert domain or the synthetic peptide containing the amphiphysin I FxDxF motif were observed primarily for residues located in β-strands 1, 4, 6, and 7 (Figure 5A and B). The shifted residues are indicated in Figure 5C in yellow ($0.1<\Delta\delta<0.2$ p.p.m.) and red ($\Delta\delta>0.2$ p.p.m.). When plotted onto the fold, these residues reveal the binding surface of the PHear domain (Figure 5D). Point mutations of amino acids predicted to be involved in FxDxF motif binding identified five residues essential for interaction (Figure 5C and E) and revealed differences in the way they contribute to the interaction. For example, binding is lost when R21, R95, and R102 are exchanged to alanine or glutamic acid, indicating a need for positive charge at these positions. Moreover, substitution of R95 by lysine promotes binding whereas the R102K substitution abolishes interaction, indicating different sterical restrictions for presentation of the positive charge (Figure 5E). Similarly, while the negative charge presented by D98 appears negligible for binding since D98E is not active, binding by D98N but not D98A or D98K suggests a requirement for a β-carbonyl group, possibly as a hydrogen bond acceptor (Figure 5E).

### *The PHear domain displays a distinct interface for FxDxF motifs*

To address if the PHear domain binds selectively to amphiphysin I or functions as a general platform for FxDxF motifs, we compared the binding properties of the conserved N-terminal regions of NECAP 1 and 2 to that of the α-ear (Figure 6A). NECAP 1 (one WxxF-acidic motif) and epsin (multiple DPW motifs) bound exclusively to the α-ear, while all proteins containing at least one FxDxF motif bound to the α-ear and NECAP 1 and 2 (amphiphysin I and II, AP180, connecdenn, Eps15, and synaptojanin 170; Figure 6A). We also used GST-tagged FxDxF motifs from various proteins

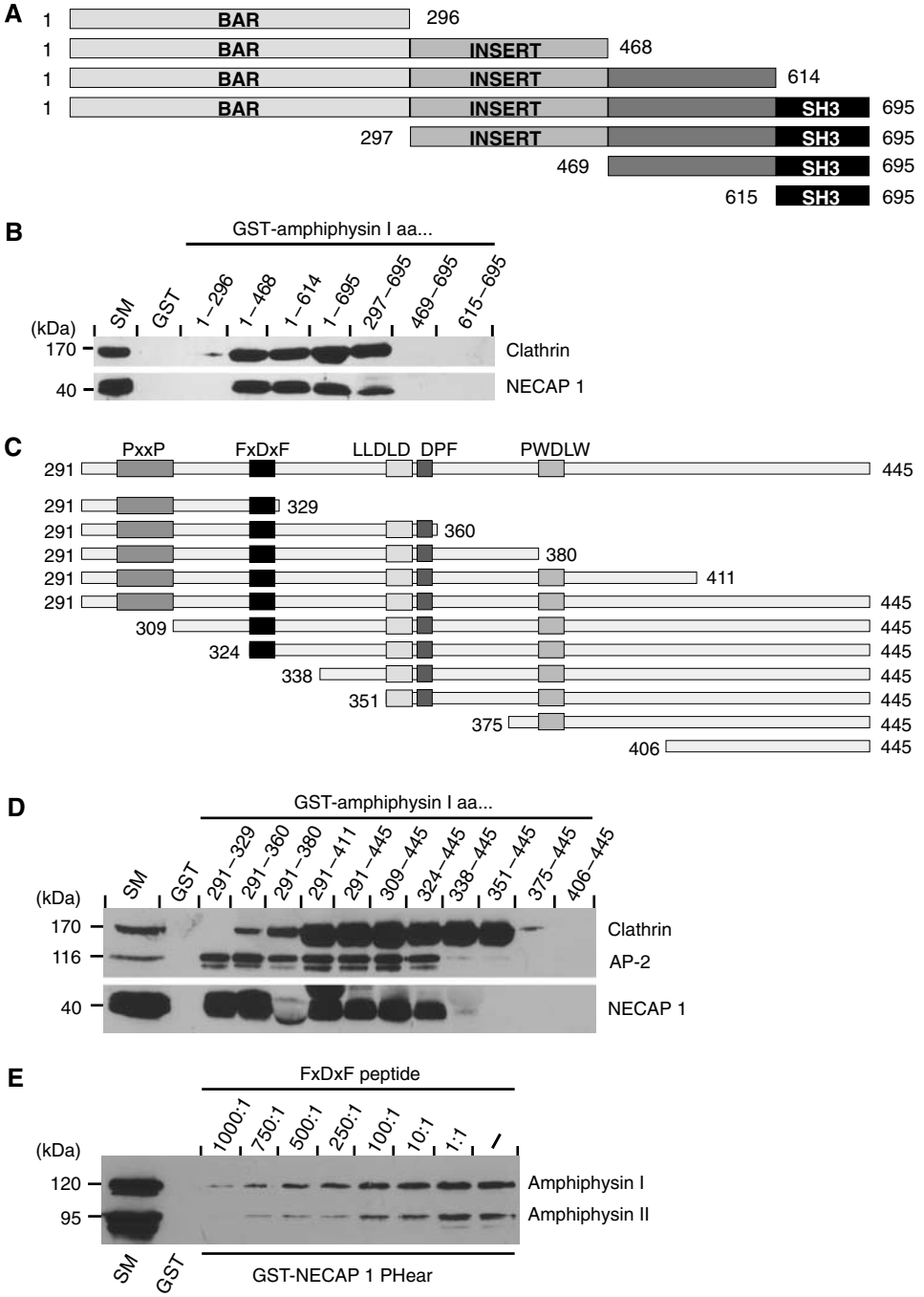



**Figure 3** Identification of the NECAP 1-binding region in amphiphysin I. (**A**) Domain structure of amphiphysin I indicating the N-terminal BAR domain followed by the neuronal insert domain, a linker region, and the C-terminal SH3 domain, and schematic presentation of N- and C-terminal deletion constructs. Amino-acid positions in front and behind the fragment indicate the protein borders. (**B**) Pull-down experiments with Triton X-100-solubilized rat brain extract using GST fusion proteins of the amphiphysin I variants indicated in panel A or GST alone pre-coupled to glutathione–Sepharose. Binding of clathrin and NECAP 1 was analyzed by Western blot. (**C**) Schematic presentation of binding motifs for endophilin A1 (PxxP), clathrin (LLDLD, PWDLW), and AP-2 (FxDxF, DPF) within the neuronal insert domain of amphiphysin I, and overview of N- and C-terminal deletion constructs with amino-acid positions indicating the protein borders. (**D**) Pull-down experiments with Triton X-100-solubilized rat brain extract using GST fusion proteins of the amphiphysin I variants indicated in panel C or GST alone pre-coupled to glutathione–Sepharose. Binding of clathrin, AP-2, and NECAP 1 was analyzed by Western blot. The faint signal for NECAP 1 with the GST fusion protein encoding amino acids 291–380 of amphiphysin I is due to co-migration of NECAP 1 with the fusion protein. (**E**) Aliquots (100 pmole) of the NECAP 1 PHear domain fused to GST or GST alone pre-coupled to glutathione–Sepharose were incubated with Triton X-100-solubilized rat brain extract in the absence or molar excess of a synthetic peptide encoding the amphiphysin I FxDxF motif (CSF**FE**D**N**F**P**E) as indicated. Binding of amphiphysin I and II was analyzed by Western blot. For all binding studies, 10% of the protein amount used in each reaction is analyzed as starting material (SM).

(amphiphysin I, AP180, disabled-2, HIP1, connecdenn, synaptojanin 170, and CALM) to pull down purified His-tagged NECAP 1 (Supplementary Figure 2). Most bound, despite the low affinity of FxDxF motifs for the PHear domain (100 μM), and the presentation of the motifs as short, unrestrained peptides. The PHear domain thus functions as a general

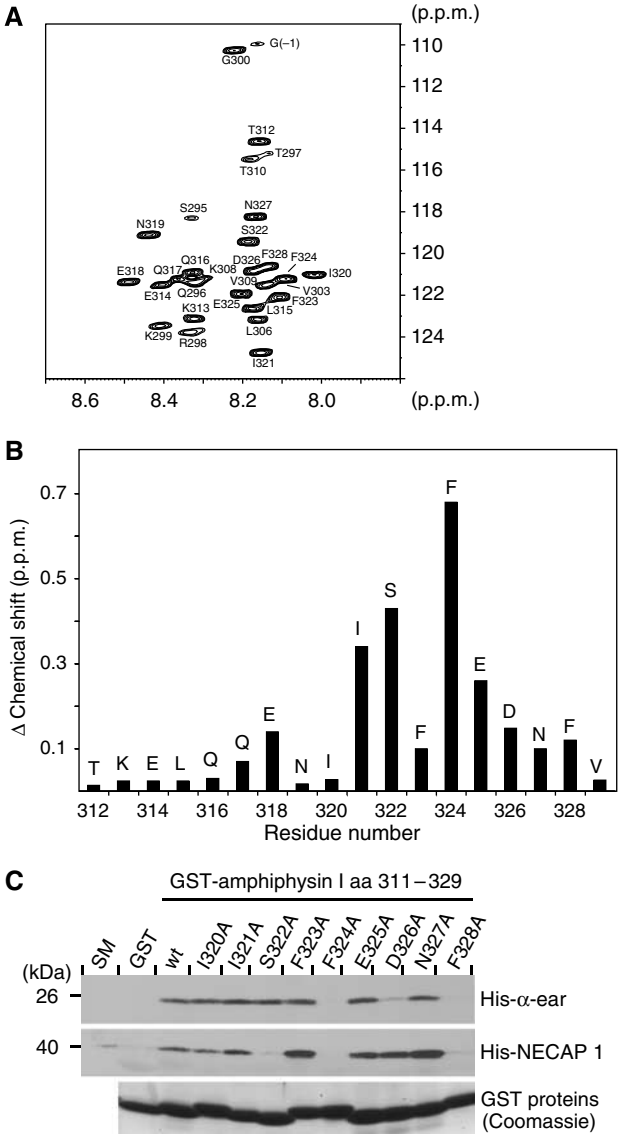



**Figure 4** The FXDXF motif in amphiphysin I mediates interaction with NECAP 1. (**A**, **B**) Mapping of amphiphysin residues involved in binding to NECAP 1. (A) $^{15}N$–$^{1}H$ HSQC spectrum of the $^{15}N$-labeled 39-amino-acid amphiphysin I peptide (amino acids 291–329) with signal assignments. (B) Magnitude of the amide chemical shift changes of the $^{15}N$-labeled 39-amino-acid amphiphysin peptide upon binding of the unlabeled NECAP 1 (the changes for N-terminal residues 291–311 residues are not presented, as their $\Delta\delta$ is less than 0.03 p.p.m.). (**C**) Pull-down experiments using purified His-tagged α-ear and His-tagged NECAP 1 and GST fusion proteins of amphiphysin I variants as indicated, or GST alone pre-coupled to glutathione–Sepharose. Binding of α-ear and NECAP 1 was analyzed by Western blot. An aliquot equal to 10% of the protein amount used in each reaction is analyzed as starting material (SM). Longer exposures reveal His-α-ear in the SM (data not shown). Aliquots of 30% of the amounts used for each GST fusion protein and GST alone were analyzed in parallel by Coomassie staining to control for protein amounts used in the pull-down experiments.

binding interface for FxDxF motif proteins. Affinity measurements by NMR revealed that the α-ear has approximately two-fold higher affinity for the amphiphysin I FxDxF motif than the NECAP 1 PHear domain (40 and 100 μM, respectively). This difference is reflected in the strength of interaction of the two domains seen for the FxDxF motif-containing binding partners (Figure 6A). Our binding studies also revealed that NECAP 1 has a higher affinity for FXDXF motif-containing endocytic accessory proteins than NECAP 2. This result was surprising due to the high degree of sequence similarity between the two isoforms within the conserved N-terminal region (Supplementary Figure 3). Sequence alignment of different NECAP orthologs, however, reveals several amino-acid exchanges between NECAP 1 and 2 located within the FxDxF motif-binding site that could account for the observed differences in affinity (Supplementary Figure 3). Despite the variation in affinity, both isoforms use the same binding site for interaction, as mutation of corresponding amino acids in both NECAP isoforms has similar effects on the binding of FxDxF motif proteins (Figure 6A, middle and right panel). The NECAPs thus interact with a variety of endocytic accessory proteins through their PHear domain and are interwoven in the endocytic protein web, raising the possibility that depletion of NECAPs would have functional implications for clathrin-mediated endocytosis. To address this issue, we used lentiviral delivery of shRNA expression constructs into COS-7 cells. For each assay, cells were co-transduced to simultaneously knock down NECAP 1 and 2, using different combinations of viruses containing shRNA sequences targeting NECAP 1 (N1nt170 or N1nt463) and NECAP 2 (N2nt266 or N2nt332). As control, cells were co-transduced with two viruses, D and Q, containing non-targeting shRNA sequences designed by Dharmacon and Qiagen, respectively. Transduced cells were readily detectable as transduction also delivered an independent GFP expression cassette. Best NECAP knockdown was achieved 5 days after transduction (data not shown), although even then, NECAP knockdown was not complete. Expression levels of RME-8, AP-2, and transferrin receptor were not affected (Figure 6B). For now, we do not know what causes the low knockdown efficiency for NECAPs, but we did not find any improvement when using other targeting sequences, changing length of transduction time or MOI, or when transfecting synthetic RNA duplexes (data not shown). However, when we tested for effects on transferrin uptake, we observed on average an approximately 40% decrease in the amount of endocytosed transferrin for cells knocked down for NECAP 1 and 2 at multiple time points (2.5, 5, or 10 min of uptake; Figure 6C and D). Quantification revealed that in nearly all conditions, NECAP 1/2 knockdown leads to a significant decrease in the amount of endocytosed transferrin (Figure 6D). These data show a functional role for NECAPs in clathrin-mediated endocytosis and support the idea that the PHear domain serves as an interaction module within the endocytic protein web, promoting efficiency of the endocytic machinery.

## Discussion

NECAPs are components of the protein machinery for clathrin-mediated endocytosis and interact directly with the AP-2 α-ear sandwich subdomain through their C-terminal WxxF-acidic motif (Ritter *et al*, 2004). The N-terminal region of NECAPs contains the PHear domain, which is a novel module in the PH domain superfamily. Other components of the endocytic machinery also contain PH superfold modules, namely PH and PTB domains, and these domains mediate specific subsets of interactions within the machinery. For

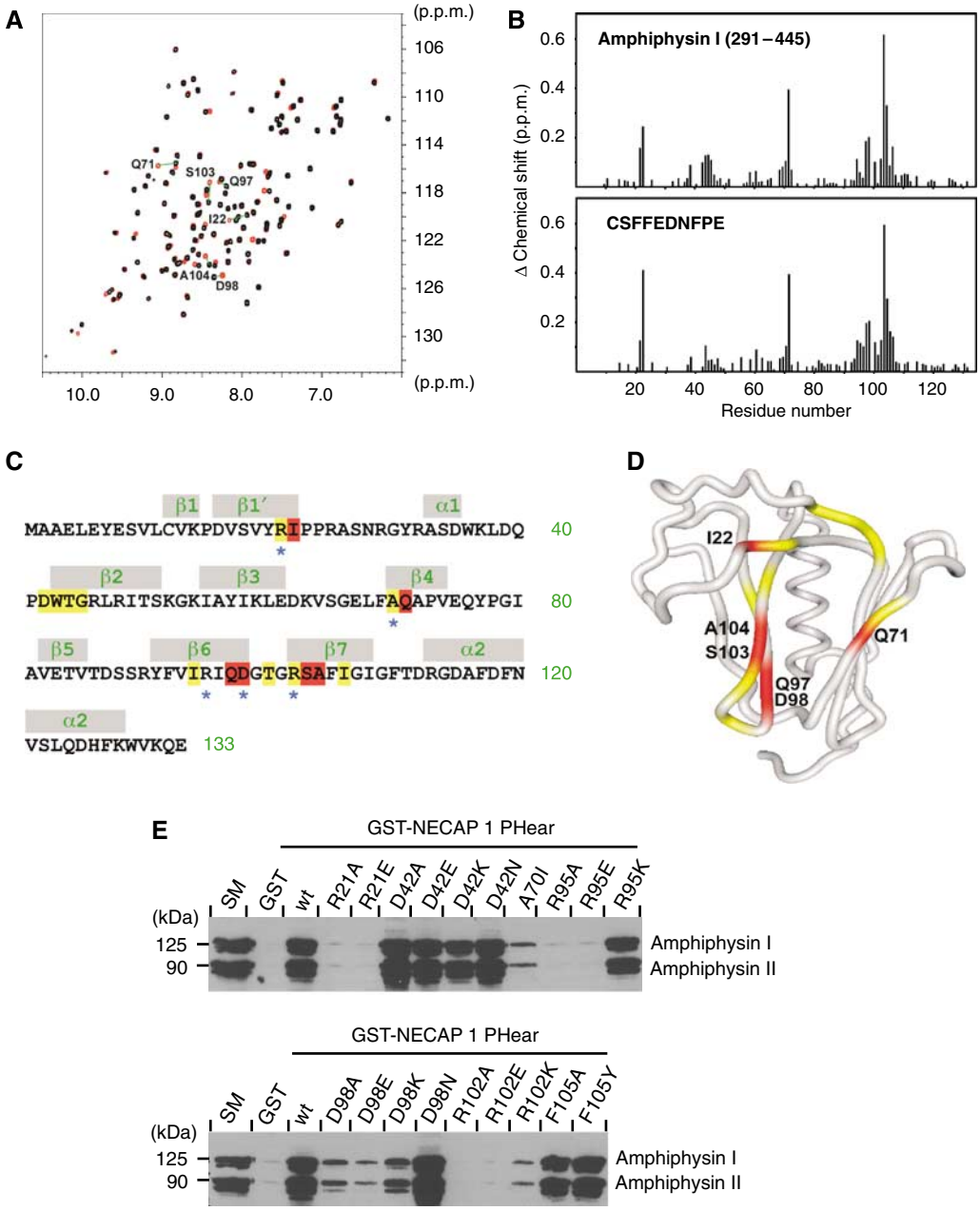
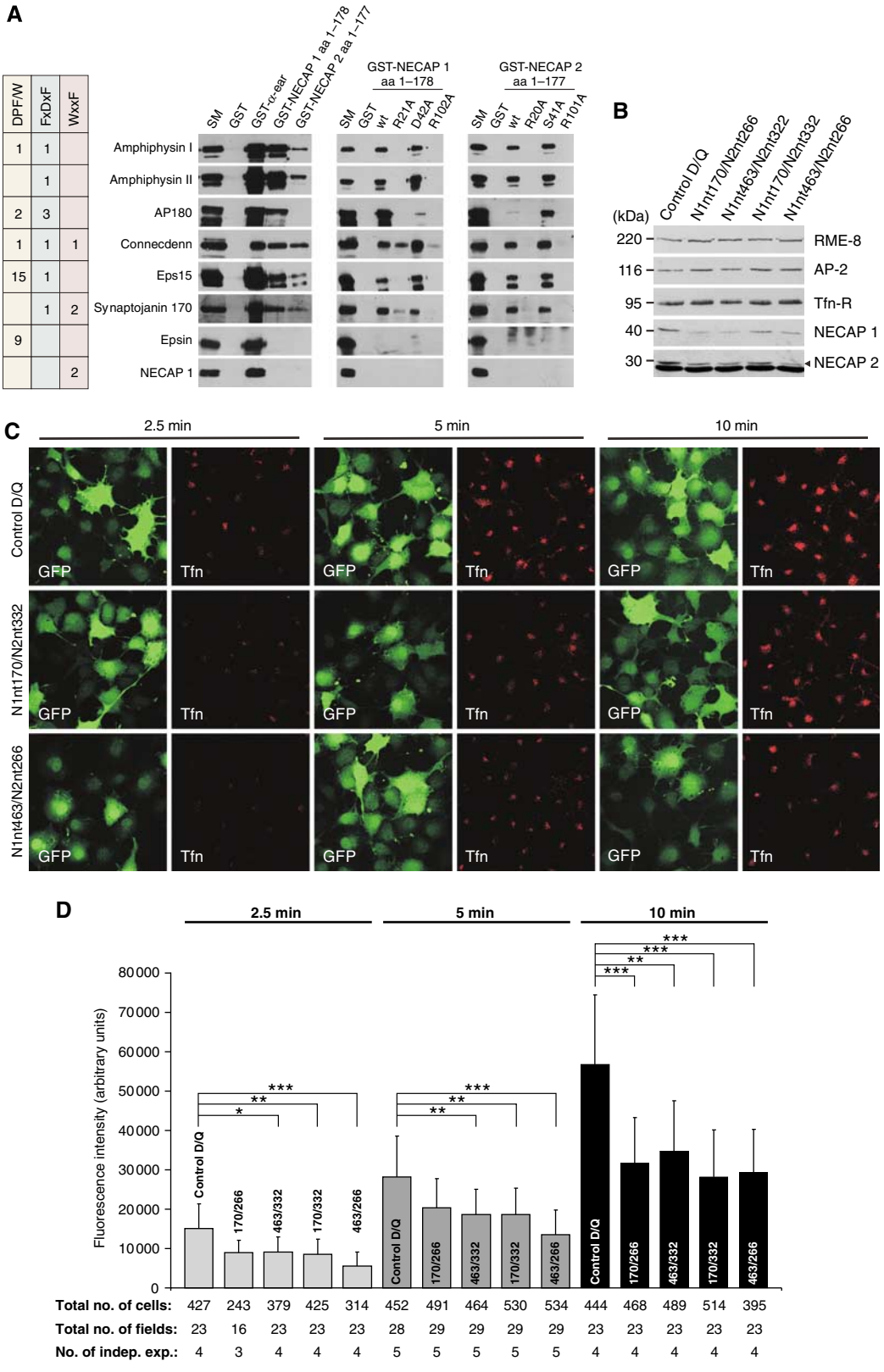



**Figure 5** Identification of the NECAP 1 binding site for FxDxF motifs. (**A**) Comparison of $^{15}N$–$^{1}H$ HSQC spectra of $^{15}N$-labeled NECAP 1 in the absence (black) or presence (red) of a synthetic amphiphysin I peptide (CSF**FE**D**N**F**P**E) (at a 2:1 peptide–protein ratio). (**B**) Magnitude of the amide chemical shift changes of the NECAP 1 residues upon binding of the amphiphysin I insert domain (amino acids 291–445) or the synthetic peptide. (**C**) Sequence of NECAP 1 with positions of β-strands and α-helices indicated. Color shading represents the size of the amide chemical shift changes (red, $\Delta\delta > 0.2$ and yellow, $0.2 > \Delta\delta > 0.1$ p.p.m.) upon binding of the amphiphysin I insert domain. Asterisks mark residues that when mutated affect binding. (**D**) Backbone trace of NECAP 1 colored according to the size of the chemical shift changes indicated in panel C. (**E**) GST fusion proteins encoding wild-type NECAP 1 PHear domain and point mutations as indicated, or GST alone pre-coupled to glutathione–Sepharose were incubated with Triton X-100-solubilized rat brain extract and interaction with amphiphysin I and II was analyzed by Western blot. A total of 10% of the protein amount used in each reaction is analyzed as starting material (SM).

**Figure 6** The PHear domain is a general FxDxF motif-binding site implicated in the regulation of clathrin-mediated endocytosis. (**A**) Pull-down experiments with Triton X-100-solubilized rat brain extract using GST fusion proteins of the α-ear and NECAP variants as indicated, or GST alone pre-coupled to glutathione–Sepharose. Binding of various endocytic accessory proteins was analyzed by Western blot and the motifs involved in α-ear and PHear domain binding are indicated. For all binding studies, 10% of the protein amount used in each reaction is analyzed as starting material (SM). (**B**) Equal protein amounts of Triton-X100-solubilized COS-7 cells transduced with various combinations of shRNA constructs for simultaneous knockdown of NECAP 1 and 2 or with control shRNA constructs (control D/Q) were analyzed by Western blot for expression levels of various proteins as indicated. The arrowhead indicates the NECAP 2 signal, the band underneath represents a cross-reaction of the antibody. (**C**) Immunofluorescence analysis for uptake of fluorescently labeled transferrin (Tfn) in COS-7 cells transduced with various combinations of shRNA constructs for simultaneous knockdown of NECAP 1 and 2 or with control shRNA constructs (control D/Q). Cells were allowed to endocytose for 2.5, 5, or 10 min, as indicated. Transduced cells express GFP from an independent expression cassette. (**D**) Quantification of the transferrin uptake assay described in panel C. The number of independent experiments (No. of indep. exp.), total number of microscopic fields analyzed (total no. of fields), and total number of cells analyzed (total # of cells) are indicated for each time-point and knockdown condition underneath the corresponding bar. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$.

example, the PH domain in dynamin binds to $PI(4,5)P_2$, contributing to membrane recruitment and the PTB domains of ARH and Dab2 bind to NPxY motifs, allowing them to function in cargo recruitment (Zheng *et al*, 1996; Klein *et al*, 1998; Morris and Cooper, 2001; He *et al*, 2002; Mishra *et al*, 2002a, b). In the case of the PHear domain, the module has evolved into a functional mimic of the α-ear platform domain and interacts with a broad class of proteins in the endocytic



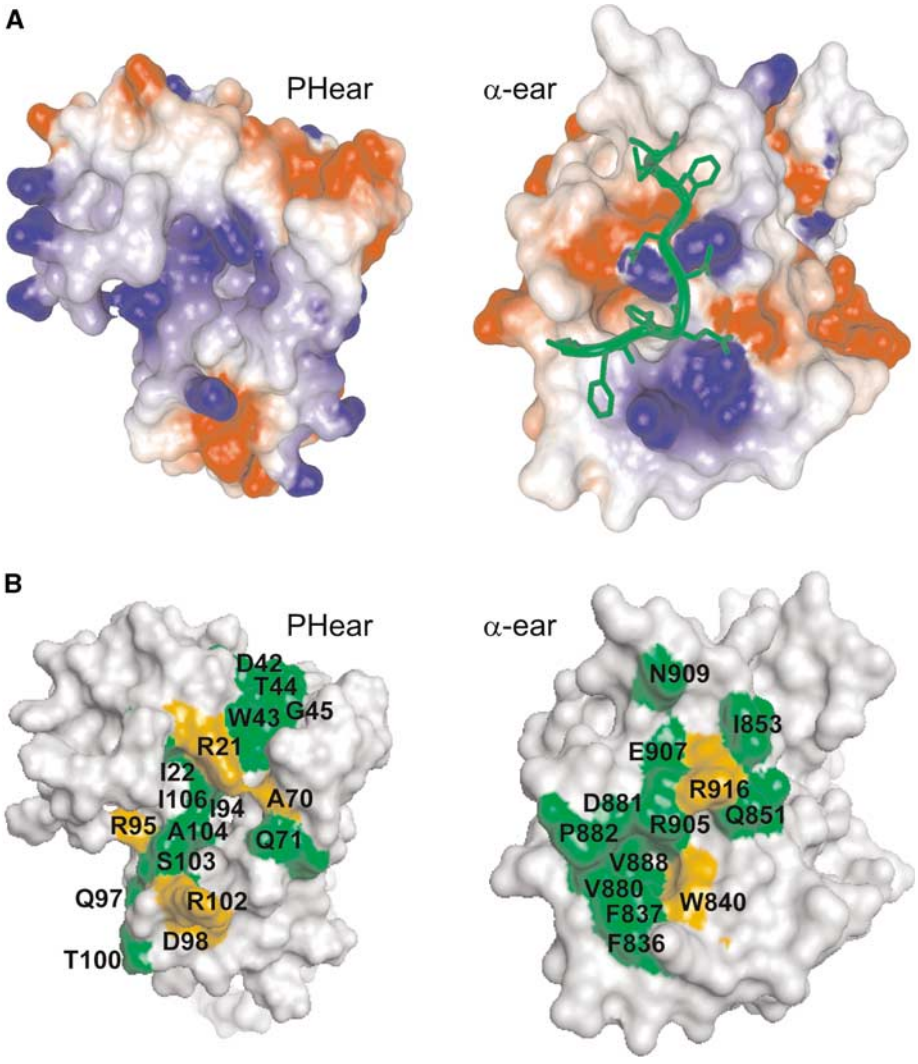



**Figure 7** Comparison of the molecular surfaces of the PHear domain and AP-2 α-ear. (**A**, **B**) Presentation of the FxDxF motif-binding surface for the NECAP PHear domain (left) and the α-ear of AP-2 (right). The orientation of the NECAP PHear domain is corresponding to Figure 5D. (A) The surface of both proteins is color-coded, with red indicating negative electrostatic potential and blue indicating positive potential. The amphiphysin I SF**FE**D**N**F**V**P peptide is shown in green. The atomic coordinates for the α-ear/FxDxF motif complex were taken from PDB entry 1KY7. (B) Color-coding (green) highlights amino acids implicated in FxDxF motif binding by NMR for the PHear domain, or shown to contact the motif by co-crystallization for the α-ear. Mutational analysis of amino acids labeled in orange verified their contribution to FxDxF motif binding (this study and Brett *et al*, 2002).

machinery. The α-ear and PHear domain share no significant sequence or structural homology and therefore expose distinct molecular surfaces for FxDxF motif binding (Figure 7A and B). This provides a likely explanation for the different requirements observed for some amino-acid positions within and surrounding the FxDxF motif core (Figure 4C), but only future extensive mutagenesis for each single position would allow the designation of α-ear- and PHear domain-specific variations of the FxDxF motif. Nonetheless, both domains target FxDxF motifs to select common binding partners. This represents an intriguing example of convergent molecular evolution, in which two distinct domains have acquired a common function, that is, to bind to FxDxF motifs. It is tempting to speculate that it results from the colocalization of the α-ear and the PHear domain within a functional microdomain.

The AP-2 α-ear recruits accessory proteins from the surrounding cytosol to sites of CCV assembly. The β2-ear also recruits accessory proteins during early steps of vesicle formation and proteins that engage α- and β2-ear simultaneously have higher avidity for AP-2. Clathrin recruitment to the maturing vesicle by the β2-ear and linker displaces the accessory proteins, many of which have FxDxF motifs, from the β2-ear (Owen *et al*, 2000; Edeling *et al*, 2006; Schmid *et al*, 2006). NECAPs target the α-ear sandwich subdomain through their C-terminal WxxF-acidic motif, thereby placing the PHear domain into the environment of the α-ear. This could provide a means to maximize the sampling radius covered by the α-ear and to increase the avidity for FxDxF motif proteins by locally increasing the number of available binding interfaces either throughout vesicle formation or at specific steps of vesicle maturation, for example, when accessory protein binding to the β2-ear is disrupted by clathrin. Knockdown of AP-2 leads to a drastic reduction of transferrin endocytosis (Conner and Schmid, 2003; Hinrichsen *et al*, 2003; Motley *et al*, 2003; Huang *et al*, 2004), attesting to

the critical role of AP-2 within the endocytic machinery. Here we observed a moderate reduction in transferrin endocytosis following NECAP knockdown. This suggests that NECAPs function supplementary to AP-2 and are involved in the fine-tuning of the endocytic machinery. In this scenario, one could imagine how NECAP knockdown could cause an imbalance in the recruitment of FxDxF motif proteins during vesicle formation, resulting in a decreased endocytic efficiency.

NECAPs also use a ΦxxΦ motif in their C-terminal region to bind the clathrin adaptor proteins AP-1 and GGAs, which function at endosomes and the trans-Golgi network (TGN) (Mattera *et al*, 2004; Ritter *et al*, 2004). The γ-ear and GAE domains of these adaptors serve to recruit accessory proteins, analogous to the α-ear (Lui *et al*, 2003). In contrast to the α-ear, however, these ears only contain a fold equivalent to the α-ear sandwich subdomain (Kent *et al*, 2002; Collins *et al*, 2003). NECAP binding to the ears of AP-1 and GGAs would provide a means to recruit FxDxF motif proteins to sites of vesicle formation at endosomes and the TGN.

The prevailing idea is that the peptide motifs that allow for targeting of endocytic proteins to the α-ear interact exclusively with this domain. The PHear domain is the first evidence that one endocytic motif can target alternative protein domains. Moreover, to the best of our knowledge, the binding of FxDxF motifs to the α-ear and the PHear domain represents the only known example where a single defined peptide motif targets two distinct protein modules. Our characterization of the NECAP PHear domain has thus revealed a surprising twist on domain/motif specificity, suggesting even more complex ways to regulate vesicle formation. Time will tell if the PHear domain alone evolved to provide an alternative mechanism of accessory protein regulation, or if this is only the first of many examples to come.

## Materials and methods

### *Expression constructs, antibodies, and peptides*

A description of the expression constructs used can be found in the Supplementary data online. Antibodies for amphiphysin I and II (1874, detects both isoforms), connecdenn, NECAP 1, NECAP 2, and RME-8 were previously described (Ramjaun *et al*, 1997; Ritter *et al*, 2003, 2004; Girard *et al*, 2005; Allaire *et al*, 2006). Monoclonal antibodies for α-adaptin and clathrin were from BD Transduction Laboratories. Monoclonal antibodies for transferrin receptor and the tetra-His tag were from Zymed and Qiagen, respectively. Polyclonal serum against Eps15 was from Santa Cruz. Antibodies against synaptojanin 170, epsin, and AP180 (F1-20) were generous gifts from Drs P De Camilli, L Traub, and E Lafer, respectively. The synthetic amphiphysin I peptide was purchased from HHMI/Keck Biotechnology Resource Laboratory, Yale University.

### *Knockdown experiments and transferrin uptake assays*

A detailed description of the shRNA constructs and the virus production procedure can be found in the Supplementary data online. COS-7 cells were plated in 24-well plates with 29 000 cells/well in regular medium. After 12–14 h, the medium was replaced with 500 μl/well of transduction medium and the two control viruses or combinations of NECAP 1 and 2 viruses were added at an MOI of 10 (for each virus). The next day, 1 ml of regular medium was added to each well. Three days after transduction, the cells were trypsinized and plated in parallel in six-well plates and poly-L-lysine-coated glass coverslips in 24-well plates (three coverslips for each virus combination). Five days after transduction, the cells in the six-well plate were analyzed by Western blot. The cells in the 24-well plate were serum-starved for at least 2 h and Alexa 546-labeled transferrin (Molecular Probes) in pre-warmed DMEM (5 μg/ml) was added at 37°C. After endocytosis was allowed to proceed at 37°C for 2.5, 5, and 10 min, the cells were chilled on ice and washed with ice-cold PBS. Surface-bound transferrin was removed by incubating the cells on ice in ice-cold acid wash (0.2 M acetic acid, 0.5 M NaCl). The cells were rinsed, fixed for 30 min on ice with ice-cold 3% paraformaldehyde in PBS, incubated 1 min in 50 mM $NH_4Cl$ in PBS, washed, and mounted for analysis on a Zeiss LSM 510 confocal microscope. Acquisition settings were kept constant for all conditions analyzed within each experiment and between experiments analyzed. For each time point and transduction condition, 5–6 pictures of randomly chosen fields of cells were taken. Transferrin uptake was quantified using NIH ImageJ software. For each field taken, all cells were outlined by hand and the total intensity of transferrin signal for the selected area was measured and divided by the number of measured cells to obtain the average intensity/cell for each field. Statistical analysis of the results was performed using the Kruskal–Wallis test, followed by Dunn's post test.

### *NMR spectroscopy and structure calculations*

GST fusion proteins of NECAP 1 (residues 1–133 and 1–178) were expressed in the *Escherichia coli* strain BL21. Cultures were grown at 37°C in M9-media supplemented with $^{15}N$ ammonium chloride and $^{13}C$-enriched glucose to produce uniformly $^{15}N$- or $^{15}N$-, $^{13}C$-labeled proteins. Following 4 h of induction with 1 mM IPTG at 25°C, GST fusion proteins were purified, cleaved with thrombin in PBS, and thrombin was removed using benzamidine Sepharose. The NMR samples contained 0.2–1.4 mM protein in 90% $H_2O$/10% $D_2O$, 25 mM sodium phosphate buffer (pH 7.2), 75 mM NaCl, 0.5 mM EDTA, and 3 mM DTT.

NMR spectra were acquired at 30°C on a Bruker DRX-600 and Varian Unity Inova 800 MHz spectrometer equipped with triple resonance probes and pulsed field gradients. The following 3D experiments were used for backbone and side-chain $^{1}H$, $^{13}C$, and $^{15}N$ resonance assignments: HNCACB, CBCA(CO)HN, HNCA, HNCO, $^{1}H$-TOCSY-(CO)HN, $^{13}C$-TOCSY-(CO)HN, $^{15}N$-edited-TOCSY, and $^{15}N$-edited-NOESY (Cavanagh *et al*, 1996). NMR spectra were processed using NMRPIPE (Delaglio *et al*, 1995) and XWINNMR (Bruker) software, and analyzed with XEASY (Bartels *et al*, 1995). Values of $^{3}J(H_NH_\alpha)$ were estimated from HNHA experiments (Kuboniva *et al*, 1994). Amide heteronuclear $^{15}N\{^{1}H\}$-NOEs were measured and used for determination of high-mobility regions of the proteins (Peng and Wagner, 1994). $^{1}H$–$^{15}N$ residual dipolar couplings (RDC) with a precision of $\pm 1.5$ Hz were extracted from (IPAP)-HSQC experiments (Ottiger *et al*, 1998) on an isotropic sample and on a sample containing 8 mg/ml Pf1-phage. The NMR assignments and values of RDC for the NECAP 1 PHear domain (residues 1–133) have been deposited in the BioMagnetic Resonance Bank under the BMRB accession number 6354.

Regions of α-helical or β-strand secondary structure were determined based on $C^{\alpha}$-chemical shifts (Wishart and Sykes, 1994), values of $^{3}J(H_NH_\alpha)$ coupling constants, and the NOE patterns (Wuthrich, 1986). The protein structure was refined using standard protocol in CNS version 1.1 (Brunger *et al*, 1998). The numbers of different types of constraints are presented in Table I. Manually assigned NOE distance constraints were classified according to the peak intensities as strong (1.8–3.0 Å), medium (2.0–4.0 Å), or weak (2.5–5.0 Å). Hydrogen bonds were given bounds of 1.7–2.3 Å (HN–O). The atomic coordinates of NECAP 1 PHear domain have been deposited in the Protein Data Bank under PDB accession code 1TQZ. The pairwise coordinate r.m.s.d. comparisons between different proteins were obtained by the DALI program (Holm and Sander, 1993). Structural images were generated with MOLMOL (Koradi *et al*, 1996) and PyMOL (DeLano, 2002).

Detailed analysis of peptide binding to the NECAP 1 PHear domain was carried out by comparison of chemical shifts for backbone amide signals in $^{15}N$–$^{1}H$ HSQC spectra. GST fusion protein of amphiphysin I residues 291–445 or 291–329 was expressed in the *E. coli* strain BL21. For 3D-NMR studies, the $^{15}N$-labeled 39-amino-acid peptide was additionally purified by reverse-phase chromatography on a C-18 column (Vydac), lyophilized, and resuspended in the buffer at pH 6.2. The $^{15}N$-edited-NOESY and $^{15}N$-edited-TOCSY experiments were used for the assignment of amide signals in the $^{15}N$–$^{1}H$ HSQC spectra at 30°C. Due to strong broadening of some HSQC signals of the 40-amino-acid peptide in complex with unlabeled NECAP 1 (in region between S322-V329), 2D chemical shift changes were measured at 1:2 protein–peptide ratio and calculated as $\{(\Delta^{1}H \text{ shift})^2 + (\Delta^{15}N \text{ shift x } 0.2)^2\}^{1/2}$ in p.p.m. for totally bound complex.

### *Binding studies*

A detailed description for the PIP strips, lipid sedimentation, and protein binding studies is given in Supplementary data.

### *Supplementary data*

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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