The FYVE domain is a conserved protein motif characterized by its ability to bind with high affinity and specificity to phosphatidylinositol 3-phosphate (PI(3)P), a phosphoinositide highly enriched in early endosomes. The PI(3)P polar head group contacts specific amino acid residues that are conserved among FYVE domains. Despite full conservation of these residues, the ability of different FYVE domains to bind to endosomes in cells is highly variable. Here we show that the endosomal localization in intact cells absolutely requires structural features intrinsic to the FYVE domain in addition to the PI(3)P binding pocket. These features are involved in FYVE domain dimerization and in interaction with the membrane bilayer. These interactions, which are determined by non-conserved residues, are likely to be essential for the temporal and spatial control of protein associations at the membrane-cytosol interface within the endocytic pathway.

Although FYVE domains bind PI(3)P efficiently in vitro, when expressed in cells, isolated FYVE domains often fail to localize to endosomes (11–14). Observations such as these have suggested that the ability of FYVE domains to localize to early endosomes may be determined by structural features distinct from those directly involved in PI(3)P binding (10). However, there is very little sequence conservation among FYVE domains in regions that do not directly contact the Ins(1,3)P2 group. These residues are localized within three conserved signature motifs, the WXXD, R/R/K/HCCR, and RVC motifs.

The FYVE domain is a double zinc finger named after the first four proteins found to contain this motif (Fab1, YotB, Vac1p, and EEA1) (1). FYVE domains bind with high specificity to phosphatidylinositol 3-phosphate (PI(3)P) (2–5), which is highly enriched in early endosomes (5). Crystallographic and NMR analyses of isolated FYVE domains and crystallographic analysis of the homodimeric C terminus of EEA1 (6–10) have revealed that six residues from the FYVE domain directly contact the Ins(1,3)P2 group. Thus, the molecular basis for the differing ability of FYVE domains to localize to endosomes remains unknown.

To resolve this question, we have systematically analyzed the cellular localization of a series of isolated FYVE domains from the mammalian proteins EEA1, Hrs, SARA, FGD1, and frabin. Beyond the FYVE domain, there are no functional or structural similarities among these proteins, and they each have been hypothesized to fulfill distinct cellular functions (13, 15–30).

With the exception of one residue in the FYVE domain of FGD1, all these FYVE domains contain the specific residues that directly contact PI(3)P, and all bind to PI(3)P in vitro. However, as shown here, the ability of these domains to interact with endosomes when expressed in intact cells varies greatly. A comparative analysis of the structural features within these FYVE domains that correlate with in vivo endosome binding reveals a crucial role for two variable regions within the FYVE domain: a stretch of 4 amino acids adjacent to the conserved positively charged RRHHCR region (the “turret loop”) and a region toward the N terminus of the FYVE domain that forms the interface between FYVE domain dimers in the crystal structure of the EEA1 C terminus (6). These structural differences among FYVE domains provide an evolutionary mechanism for controlling the extent and duration of the interaction between specific FYVE domain-containing proteins and early endosomes.

EXPERIMENTAL PROCEDURES

Construction of EGFP-FYVE Domains—The FYVE domain of human EEA1-(1305–1411) and mouse Hrs-(147–223) were inserted into pEGFP-C1 vector (Clontech) at the SalI and BamHI sites. The FYVE domain of human SARA-(589–656), mouse frabin-(549–618), and human FGD1-(717–789) were inserted into pEGFP-C1 vector at the BglII and SalI sites.

Construction of EGFP-FKBP Mutants—The ARGENTM regulated homodimerization kit was obtained from ARIAD Pharmaceuticals. Hemagglutinin (HA)-tagged-FKBP mutant (Fv-HA) was amplified by PCR and was inserted into pEGFP-C1 vector (Clontech) at the BspEI and BglII sites (pEGFP-Fv). The FYVE domain of human EEA1-(1305–1411, 1305–1411dm and 1337–1411), and mouse Hrs-(147–223) were inserted into pEGFP-Fv vector at the SalI and BamHI sites. The FYVE domains of human SARA-(589–656), mouse frabin-(549–618), and human FGD1-(717–789), as well a mutant of these, were inserted into pEGFP-Fv vector at the BglII and SalI sites. Mutations were constructed by the QuikChange mutagenesis kit (Stratagene).

Recombinant Protein Expression and Purification—The FYVE domain of SARA-(592–668) was subcloned into pMAL-c2x (New England Biolabs). The EEA1-FYVE domain (1336–1411) was subcloned into a modified pET-15b vector containing an N-terminal His tag (MGHHHHHHGGS). Constructs were transformed into BL21(DE3)-RIL cells (Stratagene), and cells were grown at 25 °C to an A600 of 0.4–0.6 and induced with isopropyl-1-thio-β-D-galactopyranoside for 16 h. The MBP fusion protein of the SARA-FYVE domain was purified using amylose agarose resin (New England Biolabs), and His fusion of EEA1-FYVE domain were purified by nickel-nitrilotriacetic acid agarose beads (QiaGen). All fusion proteins were further purified to homogeneity by anion
**Results**

Subcellular Localization of Isolated FYVE Domains—Initial analysis of GFP fusion proteins of the FYVE domain constructs

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**Fig. 1. Subcellular localization of different FYVE domains.** A, sequences of the mouse (m) and human (h) FYVE domains analyzed in this study, highlighting the conserved cysteines and residues involved in binding the Ins(1,3)P$_2$ head group. B, HeLa cells expressing the eGFP constructs of the FYVE domains shown above were imaged. Shown are 21 optical sections projected into a single two-dimensional image. All cells shown expressed similar levels of construct, as assessed by the total fluorescence intensity of the cell.
The results shown above suggest that the ability of the FYVE domain to interact with endosomes is strongly influenced by their oligomeric state. Thus, the ability of the isolated FYVE domain of SARA to associate with endosomes may reside in its ability to form oligomers without the need for regions outside the FYVE domain. To determine whether indeed the SARA FYVE domain forms oligomeric structures in live cells, we utilized fluorescence resonance energy transfer (FRET). To first determine whether FYVE domain dimers might be capable of undergoing FRET, CFP-Fv and YFP-Fv fusions of the FYVE domain of Hrs were co-expressed in Cos 7 cells. In the absence of dimerizer, these proteins were diffusely distributed in the cytoplasm, as shown for the eGFP construct in Fig. 3A. To determine whether formation of dimers in the cytoplasm would elicit FRET, cells were treated previously with LY294002, a reversible inhibitor of PI 3-kinase that inhibits FYVE domain recruitment to these membranes. Cells were then exposed to AP20187, and the fluorescence in the CFP and YFP channels was monitored quasicontinuously, as described under “Experimental Procedures” (Fig. 4). Within seconds after the addition of dimerizer, a clear decrease in fluorescence in the CFP channel was observed. No similar decrease was observed in control experiments in which cells expressing only CFP-Fv-FYVE(Hrs) were subjected to similar illumination intensities and times (not illustrated). Simultaneously, a significant increase in the ratio of YFP to CFP fluorescence was detected, suggesting that the decrease in CFP fluorescence was due to quenching of the CFP signal by the YFP fluorophore. Removal of LY294002 by three rapid successive washes caused the recruitment of Hrs-FYVE constructs to endosomes and an abrupt increase in the YFP to CFP fluorescence ratio. Although the cause of this increase is not clear, it may be due to an enhanced FRET efficiency when intramolecular motion is restricted to the two-dimensional plane of the endosomal surface. In any case, the results of this experiment indicate that FRET can be used as a measure of dimerization state of CFP- and YFP-FYVE domain constructs in the cytoplasm and on the endosomal surface.

To further substantiate this result, the unquenching of CFP fluorescence after photobleaching of YFP was monitored. Cells transfected with CFP- and YFP-Fv-FYVE(Hrs) were treated
was co-expressed with CFP-Fv-FYVE(Hrs), and cells were incubated with AP20187. This procedure resulted in the concentration of CFP and YFP on the same endosomal surface but through different FYVE domains. No unquenching of CFP fluorescence was detected with any of these combinations, indicating that non-cognate CFP and YFP FYVE domains do not elicit FRET despite their colocalization on the endosomal surface. Thus, the FRET detected when co-expressing CFP- and YFP-FYVE(SARA) most likely reflects the presence of spontaneously assembled SARA FYVE domain dimers.

To determine the stability of the oligomeric species formed by the SARA FYVE domain, histidine-tagged EEA1 and SARA FYVE domains were produced in Escherichia coli and subjected to size exclusion chromatography. Much of the SARA construct eluted as a higher order oligomer in the void volume of the column, but a distinct peak corresponding to a dimer was detected (Fig. 6). When this peak was reapplied to the column, it quantitatively eluted at the position of the dimer. In contrast, the EEA1-FYVE domain eluted as a monomer, revealing an inherent capability of the FYVE domain of SARA to form stable dimers. This property could explain the exceptional capacity of the FYVE domain of SARA to interact with the endosomal surface and may underlie the ability of full-length SARA to oligomerize (33).

**Effect of Dimerization of Isolated FYVE Domains on Their Endosomal Localization**—To determine whether the differing ability of other FYVE domains to interact with the endosomal surface resides exclusively in their ability to oligomerize, the Fv extrinsic dimerization moiety was added to additional FYVE domains. Surprisingly, only the FYVE domain of frabin displayed a behavior similar to that of Hrs in response to AP20187 (Fig. 7). The FYVE domain of a close homologue of frabin (FGD1) and that of EEA1 failed to bind to endosomal membranes in response to AP20187-induced dimerization. Although the failure of the FGD1-FYVE domain to bind could be attributed to the presence of a proline in place of the conserved tryptophan of the conserved WXXD motif (Fig. 1A), the failure of the EEA1-FYVE domain to bind cannot be explained by the lack of any of the conserved residues that directly contact the PI(3,5)P head group. In fact, the conserved WXXD, RRHCHR, and RVC motifs are identical between the EEA1 and SARA FYVE domains.

To determine the structural features that distinguish the FYVE domains of SARA and EEA1, we constructed a series of chimeras. Sequences directly N-terminal to the RRHCHR motif (the turret loop), and sequences that in the crystal structure of EEA1 form the dimer interface region, were exchanged. The subcellular localization of chimeras containing diverse combinations of 4 amino acids of the turret loops (TL) and 6 amino acids of the dimer interface loops (DI) of EEA1 and SARA is shown in Fig. 8. As shown previously, eGFP-Fv-FYVE-EEA1 displayed a cytosolic distribution both in the absence and in the presence of dimerizer (Fig. 8A, TL0/DI0). However, when 4 amino acids in the turret loop of EEA1 were replaced by those of SARA, strong binding to endosomes was observed in response to dimerizer (Fig. 8A, TL4/DI4). Furthermore, replacement of the corresponding amino acids in eGFP-Fv-FYVE-(SARA) with those of EEA1 caused the protein to shift (Fig. 8B, TL4/DI4) to a cytoplasmic distribution (Fig. 8B, TL4/DI4). These results suggest that the turret loop of SARA contributes significantly to the membrane partitioning of the SARA FYVE domain.

Replacement of 6 amino acids in the dimer interface of EEA1 with the corresponding region of SARA also resulted in significant endosomal binding in response to dimerization (Fig. 8A, TL4/DI4). When both the turret loop and the dimer interface

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**Fig. 4. FRET between CFP- and YFP-FYVE domains upon induction of dimerization.** CFP- and YFP-Fv-FYVE(Hrs) co-expressed in Cos7 cells were simultaneously imaged using a beam splitter as described under “Experimental Procedures.” Cells were treated with 20 μM LY294002 for 10 min prior to initiation of imaging. Stacks of 21 optical sections were acquired every 20 s. After 10 min of imaging, AP20187 was added. After a further 10 min, LY294002 was washed out by rapid addition and removal of 1 ml of buffer from the coverslip five times, giving rise to the noise seen on the plots at ~21 min of imaging. The top panels illustrate segments of the cell imaged at the time points shown above each set. Images are pseudocolored, where red represents highest intensity, and blue represents lowest intensity. The top row contains the YFP channel, the middle row contains the CFP channel, and the bottom row contains the ratio of YFP/CFP. Plotted are the mean intensity values of total cellular fluorescence in each channel, as well as the ratio of the values for YFP and CFP, at each time point acquired. This experiment was repeated four times with similar results.
region of the FYVE domain of EEA1 were replaced with those of SARA, the resulting construct displayed detectable endosome binding even in the absence of dimerizer and very strong binding in its presence (Fig. 8A, Tls/DIs), resembling the behavior of the FYVE domain of SARA (Fig. 8B, Tls/DIs). Thus, the extreme difference in the ability of the SARA and EEA1-FYVE domains to localize to endosomes is due to differences in the structure of both the turret loop and the dimer interface regions.

The structural basis for the effect of the turret loop on the endosomal localization of FYVE domains was further analyzed. Four amino acids in the turret loop of EEA1 were replaced one by one with those of SARA (Fig. 9A). Substitution of the second valine of the EEA1 turret loop with a lysine caused a significant enhancement in endosomal targeting. Replacement of the first serine for threonine had no further significant effect, but replacement of the first valine for phenylalanine greatly enhanced endosomal binding. Thus, the addition of a positive charge as well as of a more hydrophobic moiety significantly enhanced the avidity of the FYVE domain for endosomes. Consistent with this hypothesis, replacement of the phenylalanine residue in the turret loop of the SARA FYVE domain with a valine resulted in significant displacement from the endosomal

**Fig. 5.** FRET between FYVE domain dimers assessed by unquenching of donor after acceptor photobleaching. A, cells expressing CFP- (lower panels) and YFP- (upper panels) Fv-FYVE-Hrs and treated with AP20187. B, cells expressing CFP- (lower panels) and YFP- (upper panels) FYVE-SARA. A single stack of 21 optical sections was acquired, and YFP and CFP fluorescence was simultaneously recorded as described under “Experimental Procedures” (Before, left columns). To bleach the acceptor (YFP), the cell was then illuminated for 2 min with a 5× increased intensity of laser power at a wavelength of 514 nm. The laser was then retraced to deliver the wavelength and power used for the first acquisition, and a second stack of 21 optical sections was acquired (After, right columns). In C, the mean fluorescence intensity of CFP in the before and after images was used to calculate the donor unquenching (ratio of after/before images). Plotted is the donor recovery observed for the CFP- and YFP-FYVE pairs indicated in the abscissa. Bars are the mean, and lines are the S.E. of 9–21 cells for each pair analyzed. The statistical significance of the differences between the bars connected by the lines is indicated.

**Fig. 6.** Gel filtration chromatography of purified recombinant SARA and EEA1-FYVE domains. SARA and EEA1-FYVE domains were produced in bacteria as an MBP or His$_6$ fusion proteins. The majority of MBP SARA-FYVE eluted as a higher order oligomer with a small fraction of a dimeric species (apparent molecular mass of 110 kDa) and no detectable monomeric species. The dimeric species was reapplied to the column, and a single peak corresponding to the dimer was eluted, indicating that SARA-FYVE does not re-equilibrate either to a higher order oligomer or to a monomer. The His$_6$ constructs of EEA1-FYVE eluted as monomeric species. The oligomeric status of the FYVE domain constructs was confirmed by sedimentation equilibrium measurements (data not shown).

**Fig. 7.** Effects of dimerization on the localization of isolated FYVE domains in live cells. HeLa cells expressing the eGFP-Fv constructs of the indicated FYVE domains were imaged. Shown are 21 optical sections projected into a single two-dimensional image. Each row shows one transfected cell (expressing the construct indicated on the left) at the time points indicated after AP20187 treatment (0, 7.5, 15, 22.5, and 30 min). All cells shown expressed similar levels of construct, as assessed by the total fluorescence intensity of the cell before dimerizer treatment.

Endosomal Targeting by FYVE Domains
SARA (FYVE(SARA)) is shown in the rightmost panels. In A, HeLa cells were transfected with eGFP-Fv-FYVE(EEA1) constructs containing the TL and/or DI of either EEA1 (TLe, Dle, white boxes) or SARA (Tls, Dls, black boxes). Cells were treated with 100 nM AP20187 (lower panels) or vehicle alone (upper panels). In B, HeLa cells were transfected with eGFP-Fv-FYVE(SARA) constructs containing the TL and/or DI of either EEA1 (TLe, Dle, white boxes) or SARA (Tls, Dls, black boxes). Cells were treated with 100 nM AP20187 (lower panels) or vehicle alone (upper panels).

**Fig. 8. Role of the turret loop and dimer interface of the EEA1 and SARA FYVE domains.** The FYVE domains of EEA1 and SARA indicating the sequences of the turret loop (TL) and the dimer interface (DI). In A, HeLa cells were incubated in the absence (upper panels) or in the presence of 100 nM AP20187 (lower panels). In B, HeLa cells were transfected with eGFP-Fv-FYVE(EEA1) or SARA (Tls, Dls, black boxes) constructs containing the TL and/or DI of either EEA1 (TLe, Dle, white boxes) or SARA (Tls, Dls, black boxes). Cells were treated with 100 nM AP20187 (lower panels) or vehicle alone (upper panels).

**Fig. 9. Hydrophobic and electrostatic interactions determine the function of the turret loop.** In A, cells expressing eGFP-Fv-FYVE(EEA1) constructs containing the wild-type turret loop sequence (SVTV, leftmost panels) or mutations in indicated residues were incubated in the absence (upper panels) or in the presence of 100 nM AP20187 (lower panels). In B, cells expressing eGFP-Fv-FYVE(SARA) constructs containing the wild-type turret loop sequence (TFTK, leftmost panels) or mutations in indicated residues were incubated in the absence (upper panels) or in the presence of 100 nM AP20187 (lower panels). A control construct lacking the Fv dimerization domain (eGFP-FYVE(SARA)) is shown in the rightmost panels.

Localization (Fig. 9B), and a further significant effect was observed upon replacement of the positively charged lysine for valine. Thus, both positive charge and hydrophobicity provide the structural requirements for the enhancement of endosome binding attributable to the turret loop.

**Role of the Coiled-Coil Region of EEA1**—The reason for the failure of the FYVE domain of EEA1 to localize to early endosomes when dimerization is mediated by the Fv moiety is not clear but may be related to the unusual nature of the dimer interface in the EEA1-FYVE domain which, beyond displaying a high degree of complementarity between the interacting surfaces, lacks any stabilizing electrostatic or hydrophobic interactions (6). The precise positioning provided by the rigid coiled-coil that leads directly into the organized FYVE domain homodimer may be crucial in the case of EEA1. Alternatively, the coiled-coil may interact with specific factors at the endosomal surface. One of these factors, Rab5, has been shown to interact with the EEA1 C terminus. To test this hypothesis, constructs containing an additional 32 amino acids of the coiled-coil region without (eGFP-Fv-FYVE(EEA1<sub>1305-1411</sub>)) or with mutations that impair Rab5 binding (replacement of glutamines 1328 and 1335 for alanine, eGFP-Fv-FYVE(EEA1<sub>1305-1411(dm)</sub>)) (34) were analyzed. Both constructs displayed a mostly cytosolic distribution at steady state (Fig. 10) and were strongly shifted to the endosome upon the addition of dimerizer. These results suggest that the coiled-coil compensates for the absence of stabilizing interactions within the dimer interface.

**General Requirement for Turret Loop and Dimer Interface**—The results shown above suggest that the turret loop and dimer interface regions are generic determinants of the avidity of FYVE domains for endosomal membranes. To test this hypothesis further, we performed a structure function comparison of a different pair of FYVE domains that display vastly contrasting capacities to associate with early endosomes. The FYVE domain of FGD1 fails to bind endosomes even upon dimerization, whereas that of a close homologue, frabin, binds avidly in response to dimerizer (Figs. 1 and 7). Comparison of these two FYVE domains reveals three differences in conserved residues, where the tryptophan and aspartic acid residues of the WXXD motif are replaced by proline and glutamic acid residues, respectively, and the terminal arginine residues of the RRHHCR motif are replaced by a lysine residue. Substitution of conserved residues in the WXXD and RRHHCR motifs would be expected to diminish the specificity and affinity of PI(3)P binding, and therefore disrupt endosomal targeting, for the following reasons. First, the carboxylate group of the aspartic acid residue contacts the 5- and 6-hydroxyls of PI(3)P, whereas the aromatic side chain of the tryptophan stacks against that of the second histidine residue in the RRHHCR motif, thereby positioning the latter to interact with the 4-hydroxyl group. Second, the side chains of the conserved tryptophan, aspartic acid, and histidine residues are further predicted to sterically hinder the binding of polyphosphoinositides. Finally, the terminal arginine residue of the RRHHCR motif mediates a bivalent contact that cannot be mimicked by a lysine side chain.

To test this hypothesis, the WXXD motif in FGD1, as well as lysine 754, were replaced by the corresponding amino acids in frabin. This construct (FGD1WDR) displayed a cytoplasmic distribution in the absence of dimerizer but was recruited to endosomes in its presence, albeit to a significantly lower extent than the FYVE domain of frabin (Fig. 11). To test the hypo-
Endosomal Targeting by FYVE Domains

The direct interaction of cytoplasmic proteins with phosphoinositides is relevant for crucial cellular regulatory events, including signal transduction and membrane trafficking (Itoh and Takenawa (43); Martin (44)). Several protein modules have been found to interact directly with specific phosphoinositides including the pleckstrin homology domain, the Phox homology domain, and the FYVE domain. The structural requirements for phosphoinositide binding by these protein modules have been elucidated by crystallographic and NMR studies (6–10). However, a systematic analysis of how the structural requirements for ligand binding are related to the association of these domains with biological membranes has been missing. Such an analysis is necessary to determine the extent to which the information derived from such structural studies can be directly used for understanding the interactions of these protein domains with biological membranes in live cells.

Here we have undertaken a systematic analysis of the structural requirements for the association of isolated FYVE domains with endosomes in live cells. The results reveal that PI(3)P binding alone fails to provide a sufficient energetic contribution to drive endosomal localization. Three weak structural determinants act synergistically to establish a broad range of endosome targeting propensities. These three determinants are (a) PI(3)P recognition, mediated by highly conserved residues; (b) the turret loop, which ranges from weak (EEA1) to strong (SARA, frabin); and (c) the dimer interface loop, which also ranges from weak (EEA1) to strong (SARA). The physicochemical properties of the turret loop correlate with the ability of FYVE domains to partition with liposome membranes containing PI(3)P in vitro and with predictions of electrostatic calculations (36, 37). These structural requirements for endosome binding can be generalized to other FYVE domains. Thus, the differences in binding of the FYVE domains of frabin and FGD1 to endosomes, after equalizing those residues directly involved in contacting PI(3)P, are fully explained by differences within the 4 amino acids of their turret loops.

The requirement for multiple weak structural features for endosomal localization provides a mechanism for precisely regulating the interaction of FYVE domain-containing proteins with the endosomal surface and is likely to be highly relevant physiologically. For example, PI(3)P is likely to vary in concentration at different stages of the endosomal pathway. FYVE domains with high avidity for PI(3)P can be present at every endosomal stage, whereas FYVE domains of lower avidity may be restricted to regions of high PI(3)P concentration. It is notable that the FYVE domain of highest avidity for the endosome is that of SARA, a protein required to mediate the interaction between transforming growth factor-β receptors and the transcription factor Smad2. Transforming growth factor-β receptors are present at copies as low as 1000/cell (38, 39). The high avidity of the SARA FYVE domain would ensure the presence of high levels of SARA throughout endosomal system, enhancing the probability of its interaction with internalized transforming growth factor-β receptors. In contrast, EEA1, which is abundant in cells, has a comparatively weak FYVE
The requirement for specific quaternary structure for FYVE domain function can explain apparently discrepant results where a FYVE domain is found to be necessary for the localization of a protein to endosomes, but the FYVE domain of the same protein does not localize to endosomes when expressed alone. A typical example is that of Hrs, in which mutation of key residues in the PI(3)P binding pocket of its FYVE domain renders it cytosolic (21), and treatment of cells with wortmannin partially diminishes its endosomal localization (40), but when expressed alone, the N terminus containing the Hrs FYVE domain fails to localize to endosomes (21, 41). These studies have suggested that additional interactions independent of the FYVE domain are required for the endosomal localization of Hrs. In the studies shown here, the FYVE domain of Hrs was cytosolic but readily localized to endosomes upon dimerization. Thus, the inability of N-terminal products of Hrs to bind to endosomes could be explained by the loss of key quaternary structure rather than by the loss of other yet unidentified interactions. The formation of Hrs dimers may be influenced by the cooperativity between the FYVE dimer interface and the FYVE turret membrane interaction and may be lost upon cell lysis (41). Although elucidating the role of each FYVE domain-containing protein requires rigorous analysis of each individual case, the study presented here provides a unifying view of the structural features within the FYVE domain that are required for its function as an endosomal targeting motif.

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