

# The Cell Fate Determinant Numb Interacts with EHD/Rme-1 Family Proteins and Has a Role in Endocytic Recycling

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The adaptor protein Numb is necessary for the cell fate specification of progenitor cells in the *Drosophila* nervous system. Numb is evolutionarily conserved and previous studies have provided evidence for a similar functional role during mammalian development. The Numb protein has multiple protein-protein interaction regions including a phosphotyrosine binding (PTB) domain and a carboxy-terminal domain that contains conserved interaction motifs including an EH (Eps15 Homology) domain binding motif and  $\alpha$ -adaptin binding site. In this study we identify the EHD/Rme-1/Pincher family of endocytic proteins as Numb interacting partners in mammals and *Drosophila*. The EHD/Rme-1 proteins function in recycling of plasma membrane receptors internalized by both clathrin-mediated endocytosis and a clathrin-independent pathway regulated by ADP ribosylation factor 6 (Arf6). Here we report that Numb colocalizes with endogenous EHD4/Pincher and Arf6 and that Arf6 mutants alter Numb subcellular localization. In addition, we present evidence that Numb has a novel function in endosomal recycling and intracellular trafficking of receptors.

## INTRODUCTION

Signaling from cell surface receptors is regulated, in part, by controlling the level of receptor protein at the plasma membrane. The rapid internalization and incorporation of receptors into intracellular vesicles facilitates their removal from the plasma membrane and subsequent downregulation. Internalization occurs through either constitutive or receptor-activation-dependent pathways that can be clathrin-dependent or clathrin-independent. During clathrin-dependent internalization, receptor proteins at the cell surface interact with endocytic proteins that target them into clathrin-coated pits. Sorting to clathrin-coated pits is largely dependant on sequence motifs within the cytoplasmic domain of the receptor. The two major classes of sorting signals, tyrosine- and dileucine-based signals, are recognized by the heterotetrameric adaptor complex AP2, which recruits clathrin and nucleates the formation of the clathrin-coated vesicle (Bonifacino and Traub, 2003). In contrast, little is known about the sorting mechanisms involved in clathrin-independent internalization pathways. Nonclathrin endocytic pathways include phagocytosis, macropinocytosis, and caveolae-mediated uptake (Nichols and Lippincott-Schwartz, 2001). Receptors such as major histocompatibility complex (MHC) I, integrins, and E-cadherin are internalized independently of clathrin but require the small GTPase ADP ribosylation

factor 6 (Arf6; Donaldson, 2003). Both clathrin-coated vesicles and vesicles internalized by clathrin-independent pathways are trafficked to the tubulo-vesicular early endosome (Bonifacino and Traub, 2003). Within this compartment several groups have noted that vesicles internalized by clathrin-independent pathways converge or fuse with clathrin-derived vesicle populations (Naslavsky *et al.*, 2003; Sharma *et al.*, 2002). From the early endosome, receptors are then either routed through the recycling endosome back to the cell surface or retained and sorted into the multivesicular body and ultimately degraded in the lysosome (Katzmann *et al.*, 2002). Sorting decisions are directed by sequences intrinsic to the receptors themselves and by posttranslational modifications such as phosphorylation and ubiquitination that provide docking sites for endocytic proteins that subsequently coordinate receptor sorting and trafficking (Hicke, 1999; Katzmann *et al.*, 2001).

The adaptor protein Numb interacts with proteins involved in protein ubiquitination (Juven-Gershon *et al.*, 1998; Susini *et al.*, 2001; Nie *et al.*, 2002; McGill and McGlade, 2003) and receptor-mediated endocytosis (Santolini *et al.*, 2000; Berdnik *et al.*, 2002). Numb was originally identified in *Drosophila* (dNumb) as a mutation that caused the development of extra hair cells at the expense of sensory neurons during development (Uemura *et al.*, 1989). The Numb protein functions as an intrinsic cell fate determinant that is asymmetrically localized in neuronal precursor cells where it influences cell fate by antagonizing signaling from the Notch receptor (Frise *et al.*, 1996). Although the exact mechanism by which Numb mediates its neurogenic effect is unknown, evidence suggests that asymmetric cell divisions may in-

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involve partitioning of the endocytic machinery allowing differential control of cell surface receptor levels. Indeed, a report by Berdnik *et al.* (2002) demonstrated that the clathrin adaptor  $\alpha$ -adaptin homologue in *Drosophila* is also asymmetrically localized and  $\alpha$ -adaptin mutants show cell fate transformations similar to the Numb phenotype (Berdnik *et al.*, 2002).

Studies suggest that mammalian Numb (mNumb) functions in a similar manner to its *Drosophila* counterpart. Transgenic expression of mNumb in *Drosophila* rescues the Numb null phenotype (Zhong *et al.*, 1996), and its ectopic overexpression results in cell fate transformations identical to overexpression of dNumb (Verdi *et al.*, 1996). Mammalian Numb is asymmetrically localized in dividing neuroepithelial cells in the developing vertebrate brain (Zhong *et al.*, 1996; Wakamatsu *et al.*, 1999) and retina (Cayouette *et al.*, 2001), and homozygous deletion of Numb results in severe defects during neurogenesis, resulting in embryonic lethality around day 11.5 in utero (Zhong *et al.*, 2000; Zilian *et al.*, 2001; Petersen *et al.*, 2002). Furthermore, recent work has demonstrated that Numb overexpression antagonizes Notch receptor signaling (Berezovska *et al.*, 1999; French *et al.*, 2002; McGill and McGlade, 2003).

Both mammalian and *Drosophila* Numb proteins possess an amino terminal phosphotyrosine binding, or PTB, domain followed by a carboxy-terminal region that contains conserved sequence motifs including aspartate-proline-phenylalanine (DPF) and asparagine-proline-phenylalanine (NPF) motifs. The DPF motif is a consensus binding site for the clathrin adaptor  $\alpha$ -adaptin, whereas NPF motifs associate with proteins containing Eps15 Homology (EH) domains. Both motifs are conserved in all vertebrate Numb isoforms, in the related mammalian protein Numlike, and in the *Drosophila* homologue.

To identify Numb-binding proteins with conserved function, we conducted a parallel screen of embryonic fly and mammalian expression libraries to identify targets of the conserved sequence motifs in the Numb carboxy-terminal domain. Here we report the identification of an evolutionarily conserved interaction between Numb and the EHD/Rme-1/Pincher family of endocytic proteins and describe a novel role for Numb in endocytic recycling.

## MATERIALS AND METHODS

### Screening of Expression Libraries

*Drosophila* embryo (0–24 h) and mouse embryo (16-days) cDNA expression libraries (Novagen, Madison, WI) were plated and protein expression was induced according to the manufacturer's instructions. The nitrocellulose filters with immobilized proteins were washed several times in TBST (20 mM Tris-base, pH 7.5, 150 mM NaCl, 0.05% [vol/vol] Tween-20 and blocked overnight in blocking buffer (5% BSA [wt/vol], 1 mM DTT in TBST). The filters were then incubated overnight with blocking buffer containing biotinylated peptides (25 pmol/ml; *Drosophila* Numb peptide: ARHSTNPFISP and mammalian Numb peptide: NPSPTNPFSSDAQKAFEIEL) that were pre-conjugated to streptavidin-alkaline phosphatase (Bio-Rad, Richmond, CA). The filters were washed five times with TBST and detection was performed with a colorimetric reaction with NBT/BCIP (Roche Molecular Biochemicals, Indianapolis, IN). Positive plaques were isolated, and two rounds of plaque purification were performed. The pEXLox plasmids were isolated according to the manufacturer's directions and sequenced.

### Antibodies and Constructs

Anti-mNumbA was generated in rabbits with a synthetic peptide (TTH-PQSPSLAKQQTFPPQYE) corresponding to amino acids 538–557 as previously described (Cat. no. 07147; Upstate Biotechnology, Lake Placid, NY; Dho *et al.*, 1999). Mouse anti-Prospero was a generous gift from Gabrielle Boulianne. Rabbit anti-dEHD was raised against a GST fusion protein of the EH-domain of dEHD (amino acids 422–533). Rabbit anti-dNumb was raised against a peptide corresponding to the carboxy terminus of dNumb (sequence ISPP-KAPAQSFVQL). Other antibodies used in this study are commercially

available and include the following: anti-Flag M2 (Sigma-Aldrich, St. Louis, MO); anti-HA (Covance, Madison, WI); anti-Arf6 (Santa Cruz Biotechnology, Santa Cruz, CA); and anti-interleukin (IL) 2 $\alpha$  (Santa Cruz). Full-length expression constructs of EHD4, wild-type Numb, and Numb mutants were generated by PCR and subcloned into pFlag (Sigma-Aldrich) or pCDNA3.1–2HA in frame with the amino-terminal epitope tag. Similarly, the GST fusions were generated using PCR and cloned into pGex-4T3 (Clontech, Palo Alto, CA) with an amino terminal GST sequence. All constructs were sequenced in both directions to ensure sequences were correct (ACTG Corporation, Toronto, Ontario, Canada).

### Cell Culture and Transfections

CHO cells were grown in Ham's minimal media (Wisent, St. Bruno, Quebec) supplemented with 10% fetal bovine serum and transfected with LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA) in OptiMEM (Invitrogen) according to the manufacturer's instructions. Cells were cultured at 37°C for 24–48 h before lysis. HeLa cells were grown in DMEM (Wisent) supplemented with 10% fetal bovine serum. For siRNA experiments, a 21-nucleotide oligomer (Dharmacon, Boulder, CO) was designed to a region homologous to Numb (nucleotides 39–57). As a control, the Scramble II duplex from Dharmacon (D-001205–20) was used.

### In Vitro Binding Experiments

Recombinant GST-fusion proteins were expressed and purified as previously described (Pellicci *et al.*, 1992). Purified proteins bound to glutathione-Sepharose 4B beads (Amersham Pharmacia Biotechnol, Piscataway, NJ) were suspended in an equal volume of Nonidet-P40 (NP40) lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 100  $\mu$ M ZnCl<sub>2</sub>, 1% [vol/vol] NP40, 10% [vol/vol] glycerol) containing Complete protease inhibitors (Roche Molecular Biochemicals) and 1 mM DTT. Each of the purified GST-fusion proteins were quantitated by SDS-PAGE and Coomassie staining and compared with BSA standards. Lysates of transfected HEK293T cells were prepared in NP40 lysis buffer and incubated with  $\sim$ 1  $\mu$ g of GST-fusion protein coupled to glutathione-Sepharose 4B beads for 2 h at 4°C. The beads were washed five times with cold NP40 lysis buffer and suspended in SDS-Laemmli sample buffer. The proteins were resolved on a polyacrylamide gel and transferred to Immobilon-P (Millipore, Bedford, MA) membrane.

### Immunoprecipitations and Western Blotting

Cell lysates were prepared from transiently transfected cultured HEK293T cells by scraping 90% confluent 10-cm dishes into 1 ml lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1% [vol/vol] Triton X-100, 10% [vol/vol] glycerol, and protease inhibitors; Roche Molecular Biochemicals) and centrifuged at 14,000 rpm to pellet the insoluble material. The protein concentrations of cell lysates were determined using the MicroBCA kit (Pierce, Rockford, IL) according to the manufacturer's directions. One milligram of total cell lysate was incubated with 1  $\mu$ g antibody and 50  $\mu$ l of 20% (wt/vol) protein G-Sepharose bead slurry. The immune complexes were washed five times in NP40 lysis buffer (described above) and eluted in boiling SDS-Laemmli sample buffer. Proteins were separated by SDS-PAGE, transferred onto PVDF membrane (Immobilon-P, Millipore, Billerica, MA), and immunoblotted with primary antibodies overnight at 4°C. Bound antibodies were visualized using HRP-conjugated protein A (Bio-Rad) or goat anti-mouse in conjunction with the ECL system (Amersham Pharmacia Biotechnol).

### Immunohistochemistry

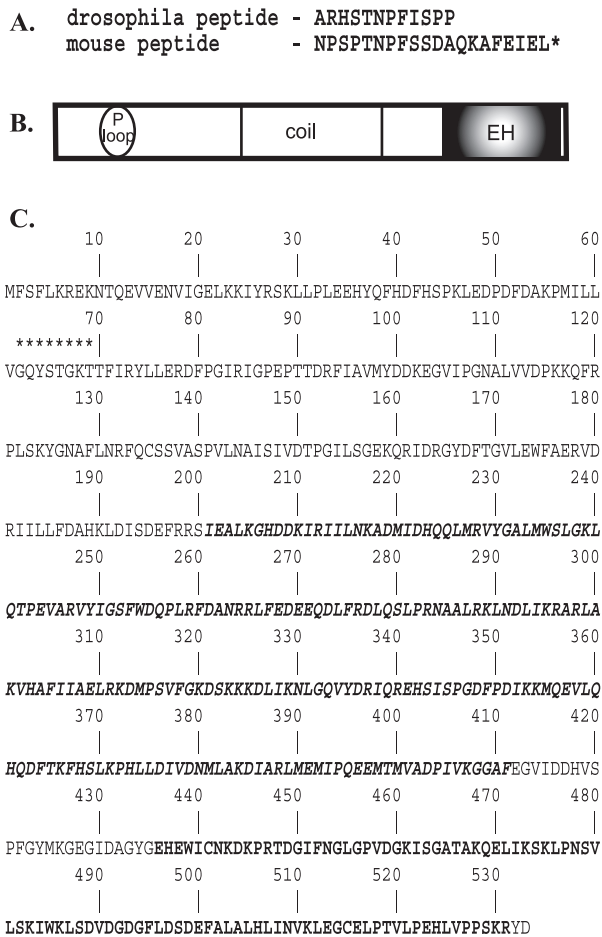
For *Drosophila* antibody staining, mixed-staged embryos were fixed in 5% formaldehyde in PEMS buffer (1 M PIPES, pH 7.2, 2 mM MgSO<sub>4</sub>, 50 mM EGTA), stained following standard procedures, and mounted in antifade (70% glycerol + 2.5% DABCO [Sigma-Aldrich] in PBS). The following primary antibodies were used: rabbit anti-dNumbC, 1:200; rabbit anti-dEHD, 1:400; and mouse anti-Prospero, 1:40. Cy3- and Alexa fluor 488-labeled secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at 1:400.

For mammalian immunostaining experiments, cells were cultured on glass coverslips. The cells were fixed in 4% paraformaldehyde (freshly prepared in PBS containing 30 mM sucrose) for 30 min at room temperature, washed with 10 mM glycine in PBS, and then permeabilized with either 0.05% saponin or 0.1% Triton X-100 for 5 min. After several short washes, the cells were blocked with either 5% BSA or 5% normal goat serum in PBS and then incubated for 30 min at 37°C with primary antibodies diluted in the blocking medium as follows: rabbit anti-NumbA 1:200, mouse anti-Arf6 1:100 (Santa Cruz) and rabbit anti-EHD4 1:100. Cy3-labeled donkey anti-mouse IgG (1:500; Jackson ImmunoResearch Laboratories) or Alexa fluor 488 goat anti-rabbit IgG (1:500; Molecular Probes, Eugene, OR) were used to indirectly label the primary antibodies. The cells were washed with PBS and mounted in DAKO mounting medium.

A modified protocol was used to costain cells with anti-NumbA and anti-EHD4, which are both rabbit polyclonal antibodies. After blocking with 5% BSA, cells were incubated with anti-NumbA, washed, and then incubated



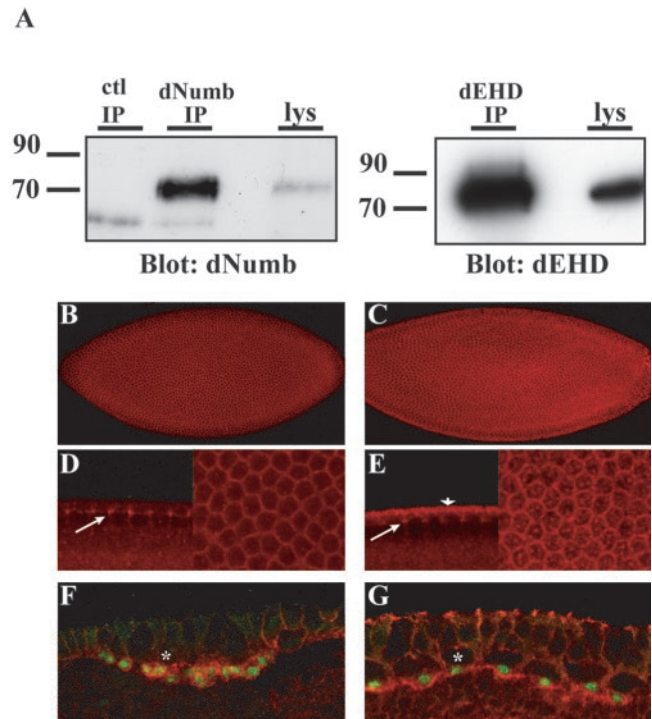




**Figure 2.** Structure of the EHD/Rme-1 family of EH-domain-containing proteins. (A) Amino acid sequence of peptides used to screen expression libraries. Peptides were coupled to biotin at the amino terminus. (B) Schematic representation of domain organization of EHD protein family. P-loop, phosphate-binding motif; coil, coiled-coil domain; EH, Eps15-Homology domain. (C) Primary amino acid sequence of *Drosophila* EHD orthologue (accession no. AF473822). Asterisks indicate the p-loop consensus sequence. The coiled-coil domain is denoted by bold italic type, and the EH-domain is indicated by boldface type.

with the early membrane insertion site identified by Lecuit and Wieschaus (2000).

One of the hallmark expression patterns of dNumb occurs within neuroblasts, the progenitor cells of the *Drosophila* CNS. The neuroectoderm of stage 8–9 embryos contains neuroblasts that divide into a large apical daughter cell that retains neuroblast characteristics and a smaller basal ganglion mother cell (GMC). During neuroblast cell division, dNumb is asymmetrically localized to the basal side of the neuroblast and the dNumb protein is preferentially delivered to the GMC (Knoblich *et al.*, 1995). Another cell fate determinant, the transcription factor Prospero is also asymmetrically segregated with dNumb into the GMC and then translocated to the nucleus and serves as a marker for the GMCs. We examined the expression of dNumb and dEHD in the embryonic neuroectoderm. As previously reported, dNumb expression was observed in Prospero-positive GMC's (Figure 3F; Knoblich *et al.*, 1995). In addition, Prospero positive GMC's were found to be enriched for dEHD

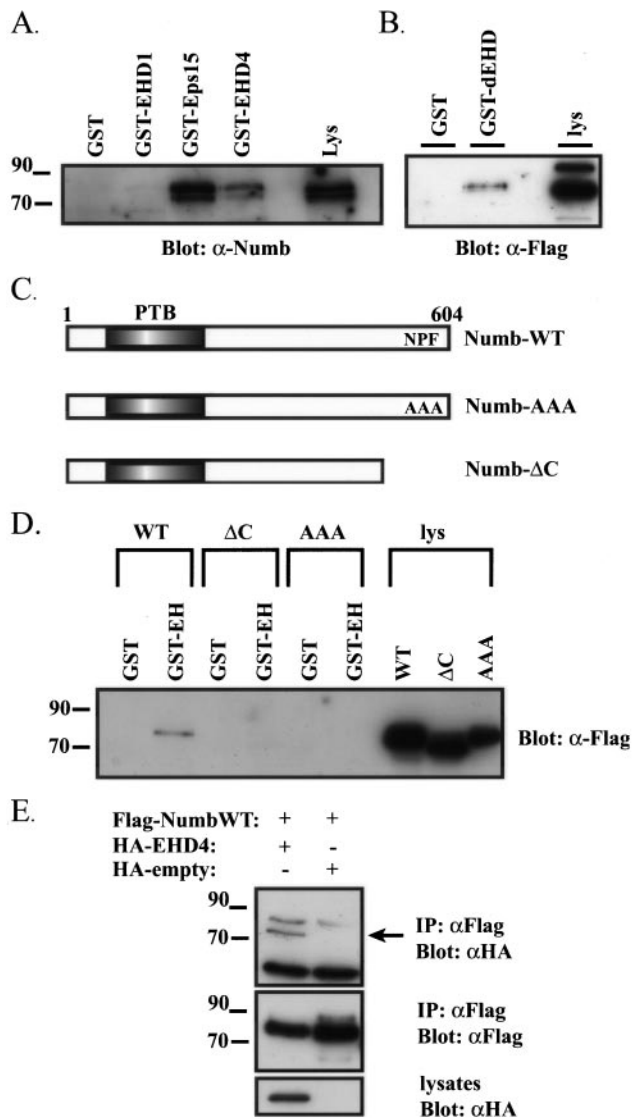


**Figure 3.** Expression pattern of dNumb and dEHD during embryogenesis. (A) Whole flies were homogenized in lysis buffer, and immunoprecipitations with either affinity-purified anti-dNumb (left panel) or anti-dEHD (right panel) were performed. Western blotting revealed a single band in both immunoprecipitation and lysate (lys) lanes corresponding to the expected molecular weight of the dNumb or dEHD protein. (B–E) Whole-mount immunostaining of stage 4–5 *Drosophila* embryos during cellularization. dNumb expression (B) is found ubiquitously in all cells. dEHD expression (C) is similarly enriched at cell borders but is also found within large vesicular like structures. Lateral and face views of B and C at higher magnification demonstrating accumulation of dNumb (D) and dEHD (E) at the progressing furrow channel (arrows, left panel) and enrichment at apico-lateral cell borders (right panels). Apical staining of dEHD (arrowhead, E) corresponds to apical membrane or apical cytoplasm. (F and G) Lateral view of whole-mount immunostaining of stage 8–9 embryos double-stained with  $\alpha$ -prospero (F and G, green) and  $\alpha$ -dNumb (F, red), or  $\alpha$ -dEHD (G, red). Asterisks demark apical neuroblast. Both dNumb and dEHD are coexpressed in Prospero-positive ganglion mother cells (GMC).

(Figure 3G) and interestingly, dEHD accumulated basally in these cells.

#### *Numb and EHD Proteins Associate In Vitro through an EH-domain-mediated Interaction*

To further investigate the interaction between mammalian Numb and EHD family proteins, GST fusion proteins of the EH-domain of EHD1 and EHD4 were produced, and Numb binding was examined. In addition, a GST fusion of the second EH-domain of Eps15 was used as a positive control. Eps15 was previously shown to associate with Numb in vitro and in vivo (Salcini *et al.*, 1997). GST-EH or GST proteins were immobilized on glutathione agarose beads and incubated with HeLa cell lysates. Endogenous Numb precipitated with the EH-domains of EHD4 and Eps15 but not with GST alone (Figure 4A). The association of the EHD1 EH-domain with endogenous Numb was barely detectable, and given that Numb binding to EHD4 was considerably



**Figure 4.** Numb and EHD proteins interact in vitro and in vivo. (A) HEK293T whole cell lysates were mixed with GST, or GST fusion with the EH-domains of EHD1 (GST-EHD1), EHD4 (GST-EHD4), or the second EH-domain of Eps15 (GST-Eps15). The complexes were washed, analyzed by SDS-PAGE, and immunoblotted with anti-NumbA (lys, whole cell lysate). (B) Cell lysates from HEK293T cells transfected with Flag-epitope-tagged *Drosophila* Numb were mixed with immobilized GST or dEHD EH-domain fusion proteins (GST-dEHD). The complexes were washed, analyzed by SDS-PAGE, and immunoblotted with anti-Flag. (lys, whole cell lysate). (C) Schematic representation of Numb and Numb mutants used to map the region of Numb responsible for interaction with EHD4. Numb-AAA contains a triple point mutation of amino acids 590–592, where the NPF (Asn-Pro-Phe) motif is mutated to AAA (Ala-Ala-Ala). Numb- $\Delta$ C is a truncation mutant that removes amino acids 567–604. (D) Immobilized GST or EHD4 EH-domain fusion proteins (GST-EH) were mixed with lysates prepared from HEK293T cells expressing Flag epitope-tagged wild-type Numb p66 (WT), carboxy-terminal deletion mutant ( $\Delta$ C), or triple point mutant (AAA). Protein complexes were washed and analyzed by SDS-PAGE and immunoblotted for Flag epitope. (E) HEK293T cells were transiently cotransfected with HA-EHD4 and Flag NumbWT. Empty vector plasmids were used in all combinations and served as internal controls to ensure binding was specific. Flag immunoprecipitations were performed and immunoblotted with  $\alpha$ -HA (top panel).  $\alpha$ -Flag immunoprecipitates is nonspecific and cross reacts with  $\alpha$ -HA

stronger, subsequent experiments focused on the Numb-EHD4 interaction.

The interaction of *Drosophila* Numb and the EH-domain of dEHD was also confirmed using in vitro binding assays. Full-length wild-type dNumb was transiently expressed in HEK293T cells, and its association with GST or the GST-dEHD was examined. Wild-type dNumb bound specifically to GST-dEHD and not control GST alone (Figure 4B), suggesting that the interaction of EHD proteins with Numb is evolutionarily conserved.

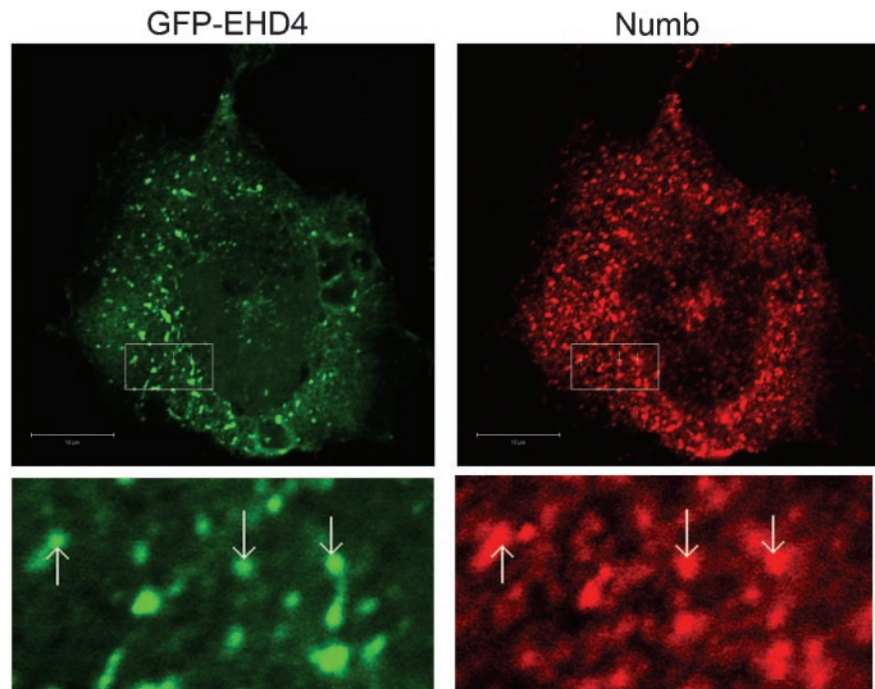
To localize the region of Numb important for the interaction with the EH-domain of EHD4, we generated a truncation mutant of Numb in which a region of the carboxy-terminal domain (amino acids 567–604) was deleted (Numb- $\Delta$ C), and a triple point mutant that converted the NPF motif (amino acids 590–592) to alanine residues (Numb-AAA; Figure 4C). GST-EH or GST proteins were immobilized on glutathione agarose beads and incubated with cell lysates containing FLAG epitope-tagged wild-type Numb or Numb mutants. Wild-type Numb bound to GST-EH; however, neither of the carboxy-terminal Numb mutants interacted with GST-EH (Figure 4D). This confirms that the formation of a Numb-EHD4 complex is mediated by an EH-domain interaction with the NPF motif in Numb.

To determine if Numb and full-length EHD4 form a complex in vivo, HEK293T cells were cotransfected with Flag-tagged wild-type Numb (FLAG-NumbWT) and either HA-tagged full-length EHD4 (HA-EHD4) or vector only (HA-empty). HA-EHD4 coimmunoprecipitated with FLAG-NumbWT (Figure 4E, top panel). Expression of FLAG-NumbWT was confirmed by stripping the membrane and immunoblotting with anti-FLAG antibodies (Figure 4E, middle panel). Similarly, whole cell lysates were immunoblotted with HA antisera to confirm the expression of HA-EHD4 in transfected lysates (Figure 4E, bottom panel).

#### *mNumb and mEHD Proteins Colocalize In Vivo*

To directly assess if Numb and EHD4 are located in the same intracellular compartments, HeLa cells were cotransfected with GFP-EHD4 and Numb and examined by immunocytochemistry. Overexpressed Numb and EHD4 were observed in both overlapping and distinct intracellular compartments. However, the degree of overlap varied across the population of transfected cells. Some cells showed very little colocalization, whereas others demonstrated marked colocalization as shown in Figure 5. Examination of optical sections near the plasma membrane did not reveal Numb and EHD4 colocalization (unpublished data). However, when midcell optical sections were analyzed, colocalization of Numb and EHD4 was observed on intracellular vesicles and tubular structures (Figure 5). In this representative field we determined that ~51% of Numb-positive vesicles contained EHD4. To examine the distribution of endogenous Numb and EHD4 proteins, MDCK and HeLa cells were costained using a modified immunohistochemical protocol. Briefly, after incubation with rabbit  $\alpha$ -NumbA, cells were incubated with excess goat  $\alpha$ -rabbit monovalent Fab fragments, followed by Alexa 488 donkey  $\alpha$ -goat secondary antibody. The second primary antibody, rabbit  $\alpha$ -EHD4, was then applied and used in

**Figure 4 (continued).** antibodies. The membranes were stripped and reblotted with  $\alpha$ -Flag to confirm the presence of Numb in the immunoprecipitations (middle panel). Similarly, whole cell lysates were immunoblotted with  $\alpha$ -HA to confirm the expression of HA-EHD4 (bottom panel).



**Figure 5.** Overexpressed Numb and EHD4 colocalize in HeLa cells. HeLa cells were transfected with pEF-Numb p66 and GFP-EHD4 and fixed 24 h after transfection, and processed for immunofluorescent localization of Numb (red) and GFP-EHD4 (green). Boxed regions have been digitally enlarged and represented as inserts in order to show more clearly examples of colabeling (arrows). In this representative section, Zeiss colocalization software compatible with Zeiss Axiovert 100 with LSM510 estimates there is ~51% colocalization of Numb and EHD4. Scale bar, 10  $\mu$ m.

conjunction with a Cy3-labeled donkey  $\alpha$ -rabbit secondary antibody. Efficient blockage of the first primary was confirmed in control preparations treated identically except for the addition of the second primary antibody. Colocalization of the endogenous proteins was observed on intracellular vesicles in both HeLa (Figure 6, top) and MDCK cells (Figure 6, middle). We noted that many of these vesicles were distinct and did not show overlapping staining; however, a proportion of vesicles were labeled by both  $\alpha$ -NumbA and  $\alpha$ -EHD4 antibodies (Figure 6). Control immunostaining experiments performed in parallel confirmed that the observed colocalization was not due to the use of two rabbit polyclonal antibodies (Figure 6, bottom).

#### Endogenous Numb and EHD4 Proteins Colocalize with Arf6

Our immunostaining experiments showed that Numb localizes to multiple endocytic structures at the plasma membrane and intracellular vesicles. To characterize the identity of Numb positive vesicles, cells were costained with markers for known subcellular compartments including  $\alpha$ -EEA1 (early endosomes),  $\alpha$ -KDEL (endoplasmic reticulum),  $\alpha$ -GM130 (*trans*-Golgi network [TGN]), and  $\alpha$ -Lamp1 (lysosomes). No significant colocalization of these markers with either Numb nor EHD4 was observed (unpublished data). Recently, EHD proteins have been implicated in the ADP ribosylation factor 6 (Arf6) regulated membrane recycling pathway (Caplan *et al.*, 2002). Arf6 belongs to a subfamily of the ras-related class of small GTPases, and cycles between the active GTP bound and the inactive GDP-bound form. This dynamic cycle is thought to mediate the regulation of a membrane recycling pathway. Previous immunocytochemistry studies demonstrated that Arf6 localized at the plasma membrane and to a distinct intracellular vesicle population which, similar to Numb, did not colocalize with known endocytic markers (Radhakrishna and Donaldson, 1997; D'Souza-Schorey *et al.*, 1998). We therefore examined whether Numb localized within the Arf6 recycling pathway, by using HeLa cells in which the Arf6 subcellular localization has been extensively characterized (Radhakrishna and Donald-

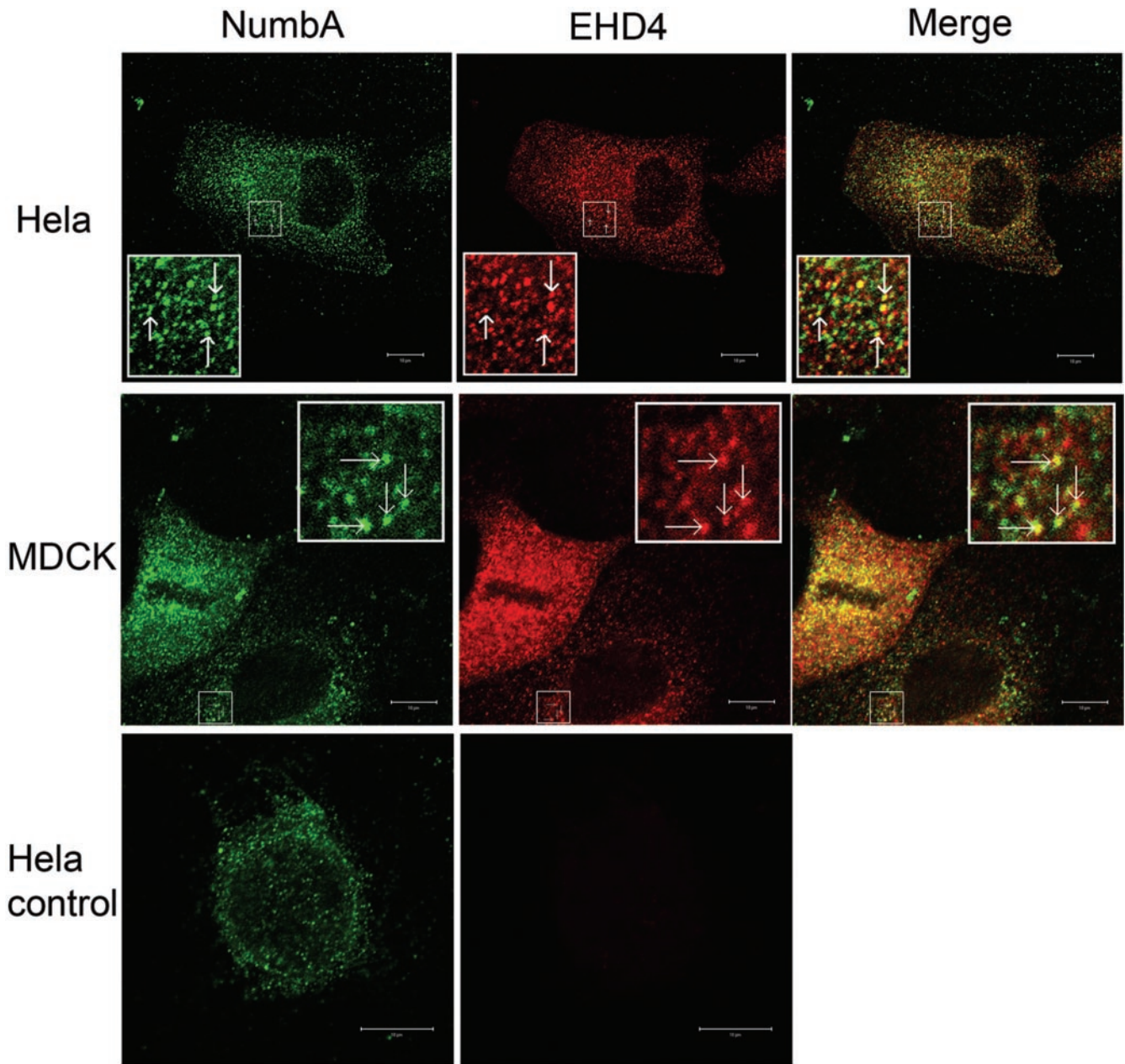
son, 1997). HeLa cells were costained with anti-Numb and anti-Arf6 antisera. Endogenous Numb and Arf6 colocalized on a subset of intracellular vesicles (~14–20%), suggesting Numb is distributed within the Arf6 recycling pathway (Figure 7, top panels). In addition, Numb and Arf6 colocalized to what appears to be the cleavage furrow (Figure 7, top panels, thickened arrow) where Arf6 was shown by Schweitzer and D'Souza-Schorey (2002) to concentrate during telophase.

Previously, Caplan *et al.* (2002) showed that Arf6 and transfected EHD1 proteins colocalize within the tubulo-vesicular recycling compartment. We therefore examined whether EHD4 shared a similar expression pattern. Similar to our observations with Numb and Arf6, EHD4 and Arf6 colocalized to intracellular vesicles in HeLa cells (Figure 7, bottom panels). Taken together these results suggest that both Numb and EHD4 are resident proteins of the Arf6-regulated membrane recycling pathway.

#### Numb Localization Is Altered by an Arf6 GDP/GTP Cycling Mutant

The use of dominant-acting Arf6 mutants that mimic either activated GTP-bound Arf6 (Arf6-Q67L) or inactive GDP-bound Arf6 (Arf6-T27N) have been instrumental in the study of Arf6-regulated membrane trafficking events. Arf6-Q67L localizes almost exclusively to the plasma membrane and its expression results in the formation of extensive plasma membrane protrusions (D'Souza-Schorey *et al.*, 1995; Radhakrishna and Donaldson, 1997). We examined the effects of Arf6-Q67L on the intracellular localization of both Numb and EHD4 co-expressed within the same cell. GFP-EHD4, Arf6-Q67L-HA, and Numb were cotransfected into HeLa cells, and their expression pattern was examined by immunocytochemistry. In cells expressing Arf6-Q67L, EHD4 colocalized with Numb and Arf6-Q67L at plasma membrane protrusions (Figure 8, top panels). In addition, Numb, EHD4, and Arf6-Q67L accumulated within macropinosomes and intracellular vacuolar clusters (Figure 8, bottom panels). In contrast, expression of the Arf6 GTP-binding defective mutant, Arf6-T27N, did not alter





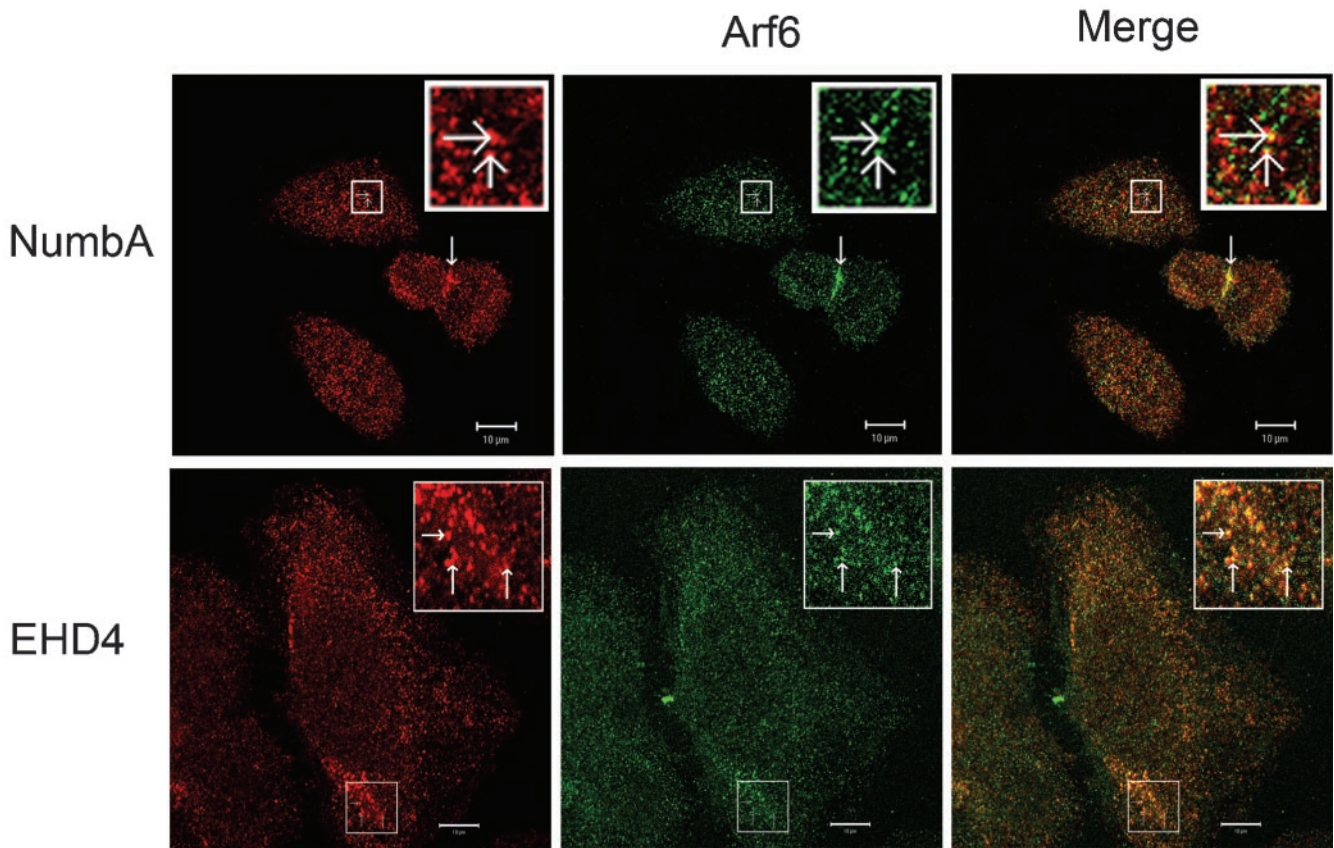
**Figure 6.** Endogenous Numb and EHD4 colocalize in intracellular vesicles. HeLa (top and bottom panels) and MDCK (middle panels) cells were costained with rabbit anti-NbA plus goat anti-rabbit Fab (green), followed by rabbit anti-EHD4 (red) as described in MATERIALS AND METHODS. The bottom HeLa cell image is a representative cell taken from a control coverslip that was treated identically to the HeLa and MDCK cells shown above it except that no anti-EHD4 was added. The parameters used for image acquisition were the same. Confocal sections taken through the middle region of the cell are presented. The red and green images were merged to show regions of colocalization of mNumb and mEHD4 (yellow). Scale bar, 10  $\mu\text{m}$ .

Numb localization (unpublished data). These observations suggest that at sites of Arf6 activation, colocalization of Numb and EHD4 is induced.

#### *Numb Functions in the Arf6-regulated Recycling Pathway*

Given the intracellular distribution of Numb and its interaction with proteins of the Arf6 recycling pathway, we tested whether Numb plays a functional role in endocytic recycling. A number of cell surface receptors are constitutively internalized and recycled via the Arf6 membrane

recycling pathway including cadherins, MHC class I, and IL-2 $\alpha$  receptor (Tac). The kinetics of Tac receptor recycling has been previously characterized and was therefore chosen as a model receptor to investigate the influence of Numb on recycling (Radhakrishna and Donaldson, 1997). The Arf6-T27N mutant has been previously shown by immunocytochemistry to inhibit recycling of receptors back to the cell surface, and we assayed the effect of this mutant to validate our biochemical assay of Tac recycling in the Arf6-dependent pathway. CHO cells were cotransfected with pTac and



**Figure 7.** Endogenous Numb and EHD4 colocalize with Arf6. HeLa cells were fixed and processed for immunofluorescence localization of endogenous Numb (top panel, red) or EHD4 (bottom panel, red), and Arf6 (green). The green and red images were merged to demonstrate colocalization of Arf6 and Numb or EHD4 (yellow). Numb and Arf6 were enriched at what appears to be the cleavage furrow (thickened arrow; top panel). Boxed regions have been digitally enlarged and represented as inserts in order to show more clearly examples of colabeling (arrows). Scale bar, 10  $\mu$ m.

either GFP-Arf6-T27N or GFP empty vector and recycling of the Tac receptor assessed by surface biotinylation (Le *et al.*, 2002). Cells were biotinylated at 0°C and then incubated at 18°C to accumulate internalized, biotin-labeled Tac receptor. The cells were MESNA stripped to remove any biotin label remaining at the cell surface and returned to 37°C to resume trafficking. At the indicated time points, cells were MESNA stripped again to remove any biotin label from endocytosed proteins that had returned to the cell surface. The cells were lysed and the unrecycled biotinylated Tac receptor was analyzed by immunoblot. After 10 min, a loss of biotinylated Tac receptor from the intracellular pool was observed in GFP-expressing cells, indicating that the Tac receptor was recycled (Figure 9A, top). As predicted, expression of the Arf6-T27N mutant severely inhibited recycling, as shown by retention of intracellular Tac receptor over time (Figure 9A, bottom).

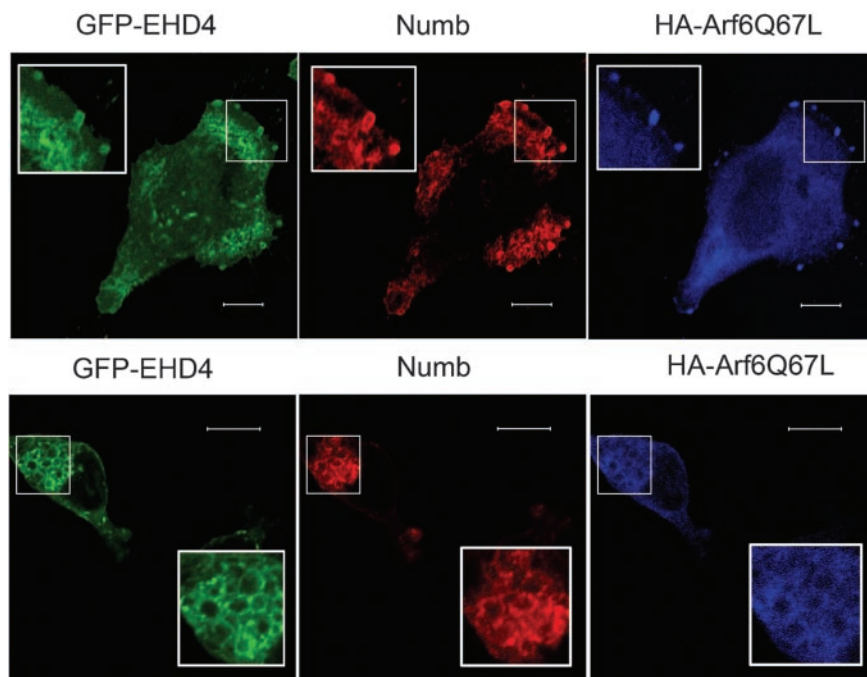
We next assessed whether reduction of endogenous Numb levels by RNA-mediated interference (RNAi) had an effect on Tac recycling. CHO cells were transfected with pTac and either control-scrambled or Numb-specific double-stranded RNA duplexes (Dharmacon), and the recycling of the Tac receptor was examined. In control-scrambled transfected cells the amount of internalized Tac receptor decreased when chased at 37°C, with maximum recycling between 3 and 10 min (Figure 9B, top). In contrast, in cells with at least 50% reduced Numb protein levels (Figure 9C, top), the amounts of labeled Tac within the protected intracellular pool remained unchanged even after 10 min, suggesting

minimal recycling (Figure 9B, bottom). Taken together these results are consistent with a role for Numb in the regulation of Tac receptor trafficking in the Arf6-dependent membrane recycling pathway.

## DISCUSSION

Our study utilized an expression screen to identify a novel interaction between Numb and EHD family of endocytic proteins in both *Drosophila* and mammals. The four members of the mammalian EHD family are highly conserved (69–72% identity) and have differential tissue expression patterns, which may define the intracellular binding partners of the individual EHD proteins (Mintz *et al.*, 1999; Pohl *et al.*, 2000). Despite extensive functional characterization of the EHD proteins, *in vivo* targets of the EH-domain have, until now, remained elusive. We have confirmed the interaction *in vitro* and *in vivo* and mapped the interaction site to the NPF motif of Numb. NPF-EH-domain interactions occur with relatively low affinity (Santolini *et al.*, 1999), and the *in vivo* interactions mediated by the EH-domain of EHD proteins are likely transient and highly regulated. Caplan *et al.* (2002) demonstrated that EHD proteins are continuously cycling on and off membranes, which is indicative of a dynamic regulatory process (Caplan *et al.*, 2002). This may in part explain why we were able to observe a direct association *in vitro*, but were unable to isolate an endogenous Numb-EHD4 complex by coimmunoprecipitation. Furthermore, colo-





**Figure 8.** Activation of Arf6 alters Numb subcellular localization. HeLa cells cotransfected with pEF-Numb p66, GFP-EHD4, and Arf6-Q67L-HA were fixed, permeabilized, and processed for immunofluorescence. Expression of Arf6-Q67L-HA (blue) induces localization of Numb (red) and EHD4 (green) to Arf6-positive membrane protrusions (top panels) and macropinosomes/vacuolar clusters (bottom panels). Boxed regions have been digitally enlarged and represented as inserts in order to show more clearly examples of colabeling. Scale bar, 10  $\mu$ m.

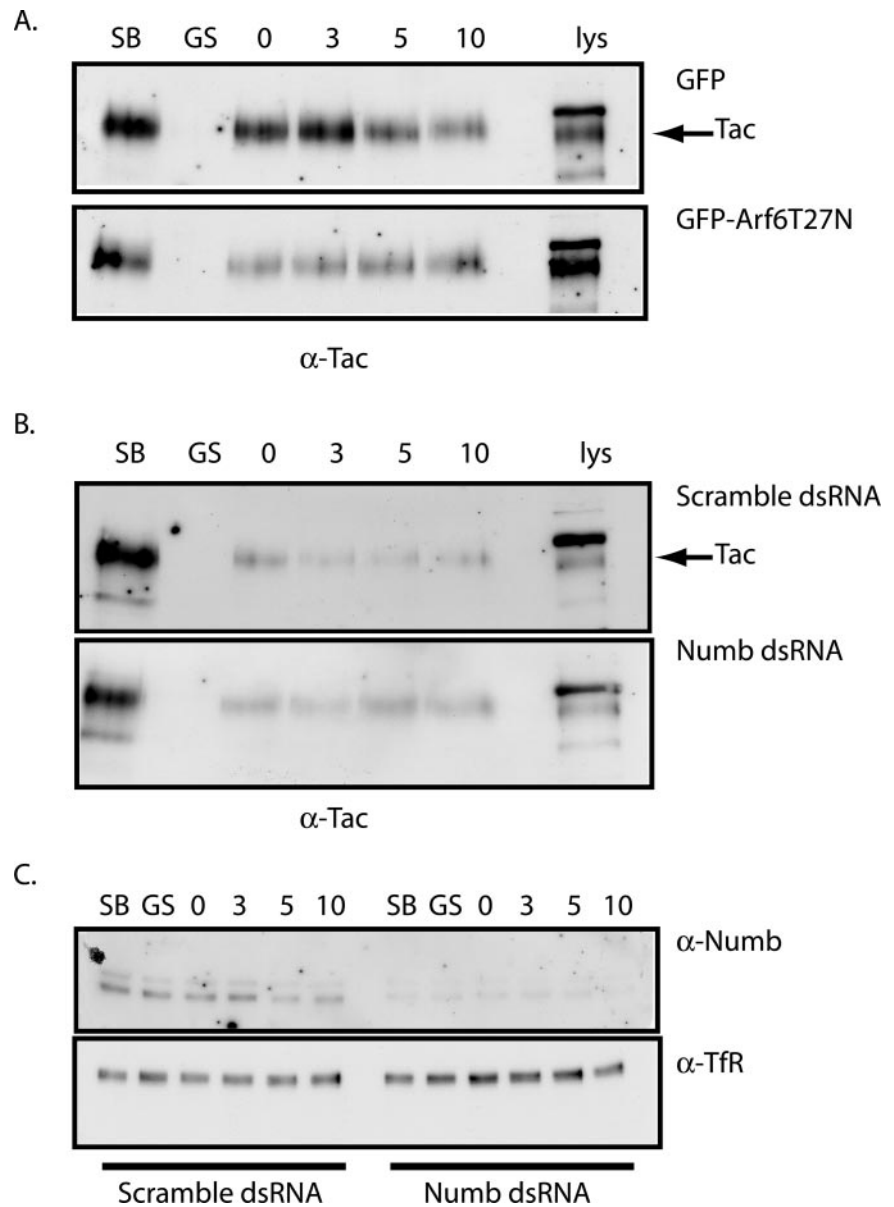
calization of endogenous Numb and EHD4 was variable across a given cell population, suggesting that the level of Arf6 activation within the cell induces the functional association of Numb and EHD4. In support of this, we observed that expression of the constitutively active Arf6 mutant induced a dramatic increase in Numb, EHD4 and Arf6 colocalization.

Recently several groups have established that EHD/Rme1 proteins function within a tubulo-vesicular endocytic recycling compartment (Caplan *et al.*, 2002; Galperin *et al.*, 2002). Both the *C. elegans* and mammalian orthologues have been characterized and were found to promote recycling of plasma membrane receptors such as the transferrin receptor in CHO cells (Lin *et al.*, 2001) and the yolk receptors in *C. elegans* (Grant *et al.*, 2001). In addition to a role in recycling back to the cell surface, EHD/Rme-1 proteins also regulate traffic from the recycling compartment to the TGN (Caplan *et al.*, 2002). This suggests that the EHD proteins may be involved in the formation of transport vesicles within the recycling compartment. EHD/Rme-1 was also found to promote recycling of receptors internalized by the clathrin-independent Arf6 endocytic pathway (Caplan *et al.*, 2002).

Previously, mammalian Numb was shown to partially colocalize with AP2 and Eps15 in clathrin-coated pits and early endosomes (Santolini *et al.*, 2000). In addition, mNumb has been shown to interact with components of the endocytic internalization machinery, AP2 and Eps15 and fragments of Numb inhibited internalization of the EGF and transferrin receptors, implicating Numb in clathrin-dependent endocytosis (Salcini *et al.*, 1997; Santolini *et al.*, 2000). We have also observed a subset of Numb colocalized at the plasma membrane with AP2 (unpublished data). However, we have found that a significant proportion of Numb is localized to intracellular vesicles that do not contain AP2 and that a subset of this vesicle population contains Arf6 and EHD4. Furthermore, we have shown that knock down of Numb by RNAi inhibits recycling of Tac; a receptor trafficked via a clathrin-independent pathway regulated by Arf6. This indicates that Numb functions in multiple steps of the endocytic pathway and reveals a role for Numb in trafficking events subsequent to internalization.

Arf6 has been predominantly associated with clathrin-independent trafficking events; however, it has also been shown to affect clathrin-dependent internalization of the transferrin receptor, in a cell type-dependent manner (D'Souza-Schorey *et al.*, 1995). Furthermore, proteins internalized by clathrin-dependent endocytosis and the Arf6 pathway have been shown to converge within the early sorting endosome (Naslavsky *et al.*, 2003). The EHD family of proteins, like Arf6 and Numb, are also found to be involved in both clathrin-dependent and -independent processes. For example, expression of EHD1 mutants disrupt transferrin receptor recycling (Lin *et al.*, 2001), but not its internalization in TRVb1 cells, whereas EHD4/Pincher was previously shown to promote clathrin-independent trafficking of the TrkA receptor in PC12 cells (Shao *et al.*, 2002). Therefore Numb, like Arf6 and EHD proteins, likely functions in multiple locations along the endocytic pathway and may represent a common component of both the clathrin-dependent and -independent vesicle-routing pathways that coordinates the trafficking of cargo between these two compartments.

Although we have shown that perturbation of Numb protein levels can affect recycling or trafficking of receptors within the Arf6-regulated membrane recycling pathway, it is not yet clear if Numb has a general or cargo-specific trafficking function. Members of the Disabled family of adaptor proteins, Dab1 and Dab2, share structural features with Numb proteins, including an amino-terminal PTB domain and a carboxy-terminal domain containing DPF motifs that interact with AP2 (Morris and Cooper, 2001). Disabled proteins have been described as cargo-specific adaptors that endocytose members of the LDL receptor family (Gotthardt *et al.*, 2000; Mishra *et al.*, 2002). There is also evidence that Dab proteins may modulate the trafficking of other transmembrane receptors. For example, Dab2 was shown to link the transforming growth factor  $\beta$  (TGF- $\beta$ ) receptor to the downstream Smad pathway (Hoccevar *et al.*, 2001). Interestingly Smad2/3 activation and signaling occurs only following TGF- $\beta$  receptor internalization into an endocytic compartment (Penheiter *et al.*, 2002). In a similar manner, Numb proteins could exert its effects on Notch signaling by specifically modifying the interaction of Notch



**Figure 9.** Numb functions in regulation of Tac recycling. (A) CHO cells cotransfected with pTac and either GFP-Arf6-T27N or pEGFP control vector were surface-biotinylated on ice for 40 min. One plate was removed to depict total surface biotinylation (lane SB), and another was stripped with glutathione to ensure efficient removal of biotin label (lane GS). The cells were then allowed to internalize for 60 min at 18°C and returned to ice to strip any label remaining at the surface. After glutathione stripping, cells were returned to 37°C for chase periods 0, 3, 5, and 10 min. At each chase time point, cells were glutathione-stripped, and remaining biotinylated proteins were recovered from equal amounts of protein lysates using streptavidin beads and were analyzed by SDS-PAGE. Tac receptor was detected by immunoblotting. Arrow indicates band corresponding to the Tac receptor. (B) Similar to experiment above, CHO cells were cotransfected with pTac and either double-stranded RNA duplex specific for Numb (bottom panel) or with a scrambled RNA duplex (top panel), and intracellular Tac receptor levels were monitored by surface biotinylation. Arrow indicates band corresponding to the Tac receptor. (C) Equal amounts of protein lysates were analyzed by SDS-PAGE to confirm depletion of endogenous Numb levels by immunoblotting for Numb (top panel). The blots were then stripped and reprobed for endogenous transferrin receptor to confirm equal loading in of each condition ( $\alpha$ -TfR, bottom panel).

with the endocytic machinery. Therefore, Numb and Disabled proteins may be members of a distinct family of endocytic adaptors with specialized functions in the regulation of signaling pathways.

In this report, we identify the *Drosophila* orthologue of the EHD/Rme1 family of endocytic proteins as the first described target of the NPF motif in dNumb. Although further genetic analysis will be required to establish the function of *Drosophila* EHD, our results strongly suggest that Numb-mediated regulation of Notch signaling may involve an Arf6-regulated trafficking pathway. A *Drosophila* orthologue of Arf6 has recently been identified (Chen *et al.*, 2003); however, its function with respect to endocytosis and vesicle trafficking has not been characterized. Given that the functional relationship between EHD and Arf6 proteins was recently confirmed in *C. elegans* (Dang *et al.*, 2004), the identification of the *Drosophila* EHD protein will facilitate further studies of the Arf6-dependent membrane trafficking pathway.

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