A Novel Transmembrane Protein Recruits Numb to the plasma Membrane during Asymmetric Cell Division

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Running Head: Membrane recruitment of Numb by NIP
ABSTRACT

Numb, an evolutionarily conserved cell fate-determining factor, plays a pivotal role in the development of Drosophila and vertebrate nervous systems. Despite lacking a transmembrane (TM) segment, Numb is associated with the cell membrane during the asymmetric cell division of Drosophila neural precursor cells and is selectively partitioned to one of the two progeny cells from a binary cell division. Numb contains an N-terminal phosphotyrosine-binding (PTB) domain that is essential for both the asymmetric localization and the fate-specification function of Numb. We report here the isolation and characterization of a novel PTB-binding, Numb-interacting protein (NIP). NIP is a multipass transmembrane protein that contains two PTB-binding, NxxF motifs required for the interaction with Numb. In dividing Drosophila neuroblasts, NIP is colocalized to the cell membrane with Numb in a basal cortical crescent. Expression of NIP in Cos-7 cells recruited Numb from the cytosol to the plasma membrane. This recruitment of Numb to membrane by NIP was dependent on the presence of at least one NxxF site. In Drosophila Schneider 2 cells, NIP and Numb were colocalized at the plasma membrane. Inhibition of NIP expression by RNA interference released Numb to the cytosol. These results suggest that a direct protein-protein interaction between NIP and Numb is necessary and sufficient for the recruitment of Numb to the plasma membrane. Recruitment of Numb to a basal cortical crescent in a dividing neuroblast is essential for Numb to function as an intrinsic cell-fate determinant.
INTRODUCTION

Asymmetric cell division, which can involve extrinsic and/or intrinsic factors, is a fundamental mechanism of generating cell diversity during the development of complex organisms (1, 2). Extrinsic factors such as Delta and its receptor Notch (3, 4), function in cell-cell communication to specify the fate of cells (5-7). Asymmetric determinants are intrinsic factors that are selectively segregated into one of the two daughter cells when a cell divides (2, 8). Consequently, the sibling cell that inherits the asymmetric determinants adopts a different fate from the one that doesn’t. Numb is a member of a growing family of proteins, which include also Prospero, Miranda, Inscuteable, and Partner of Numb (PON) (9-14), that act as intrinsic determinants in asymmetric cell division. These proteins were identified through their requirement in the development of Drosophila peripheral and central nervous systems. The external sensory organ in Drosophila is composed of two outer (hair and socket) cells and three inner (sheath, glial and neuron) cells, which are derived from a single sensory organ precursor (SOP) through three consecutive asymmetric cell divisions. Numb is selectively partitioned to one of the two daughter cells at each binary division (15). Numb is also required for the development of the central nervous system (16-18). During delaminating from the neuroectoderm and asymmetric division of a neuroblast (NB), Numb, Prospero and several other proteins are co-localized in a basal cortical crescent. These proteins are partitioned to the basal daughter cell or the ganglion mother cell (GMC) that will divide once more, generating two neurons or a neuron and a glial cell. The apical daughter to which the proteins were not partitioned to maintains the neuroblast characteristics and is capable of undergoing several additional rounds of cell division (18).
The amino acid sequence of Numb suggests that it may function as an adaptor protein capable of mediating protein-protein interactions (19). The N-terminal portion of Numb contains a phosphotyrosine-binding (PTB) domain, a protein-protein interaction module important in receptor tyrosine kinase signaling (20, 21). The C-terminal part of the protein contains a proline-rich region that can potentially interact with proteins containing the SH3 domain (22). Genetic and biochemical evidence from *Drosophila* suggest that Numb may interact with Notch to antagonize its function such that the Notch-mediated cell-cell interaction is asymmetric (17, 23). Similarly, the mammalian Numb homologue, mNumb, physically interacts with mouse Notch1 (24), and negatively regulates Notch1 activity by promoting its ubiquitination and degradation (25).

In addition to Notch, Numb interacts through its PTB domain with PON, Numb-associated kinase (NAK), and ligand of Numb X (LNX) (14, 26, 27). PON is an adaptor protein required for the basal localization of Numb during the asymmetric division of a neuroblast along the apical-basal axis (14). NAK is proposed to be a Numb-associated Ser/Thr kinase that negatively regulates Numb function (26). LNX is a unique protein containing a ring finger and multiple PDZ domains that acts as an E3 ligase for the ubiquitination and degradation of mNumb (28). The identification of this array of binding partners for Numb reflects the promiscuous nature of its PTB domain. Unlike the majority of PTB domains that require tyrosine phosphorylation for binding, the Numb PTB domain is capable of binding unphosphorylated sequences, some of which are devoid of Tyr residues (29-31). For example, the Numb PTB-binding site in NAK contains an NMSF motif (26) whereas the highly conserved mNumb PTB domain binds to an unphosphorylated NPAY-containing sequence in LNX (27, 28).

Although our understanding of the mechanism of Numb function as an intrinsic cell-fate
determinant has advanced tremendously with the identification of these Numb-binding proteins, a fundamental question remains unanswered. How is the cytoplasmic protein Numb recruited to the cell membrane in a basally localized crescent within a neuroblast, which is a prerequisite for the specific partitioning of Numb into one of two daughter cells at cell division? Since Numb lacks a transmembrane segment, its recruitment to the membrane would necessarily involve one of several possible mechanisms – by binding to a membrane lipid(s), by directly or indirectly interacting with a membrane protein, or by engaging a protein transporting machinery (32, 33). Asymmetric localization of Numb in neuroblasts requires the PTB domain (34). The pivotal role of the PTB domain is further underscored by the observation that it is indispensable not only for Numb localization during SOP and NB division, but also for the specification of sibling cell fates (34-36).

The broad specificity of the Numb PTB domain suggests that additional binding proteins may exist that localizes Numb to the plasma membrane. To search for these proteins, we screened a Drosophila embryonic cDNA library using an isolated Numb PTB domain as a probe, and identified a binding protein that we designated Numb-interacting protein (NIP). NIP is an intrinsic membrane protein that interacts specifically with Numb in vitro and in vivo, and is required for the membrane recruitment of Numb. Proteins homologous to Drosophila NIP were identified in various other species ranging from Anopheles to Homo sapiens, suggesting that the function of NIP is evolutionarily conserved.

**MATERIALS AND METHODS**

*Expression Library Screening and Cloning of NIP*- The Drosophila Numb PTB domain was purified from E.coli as described (31). Purified PTB domain was labeled with biotin using the
biotin-LC-NHS reagent (Pierce) and then used to screen a 22-24 hour Drosophila embryonic cDNA library constructed with the λEXlox vector (Novagen) following the manufacturer’s protocols. Clones displaying positive binding to biotin-PTB were identified using streptavidin conjugated alkaline phosphatase (Bio-Rad) followed by a phosphatase reaction using NBT/BCIP as substrates. The plasmids were isolated for DNA sequencing.

From 500,000 phage plaques in the primary screening, a total of 9 positives were identified, of which four displayed extensive overlaps with one another and were apparently derived from a single gene. The longest open reading frame (ORF) from these four clones encodes a 190-residue fragment of a protein. This sequence was used to search the *Drosophila* expression sequence tag (EST) database and several EST clones were retrieved. The longest EST clone (LD14488, Invitrogen) contained an ORF that encoded a protein of 474 amino acids, and the coding sequence was PCR-amplified and subcloned into a pOT2 vector (Invitrogen).

* Constructs and Antibodies - Full-length *NIP* cDNA was subcloned by PCR amplification into pEGFP, pFLAG-CMV2, or pcDNA3 vectors as required. Fragments encoding various truncated forms of NIP, eg., NIP-N(residues 1-106), -N1(residues 1-148), -N1/2(residues 1-382), -N2(363-474aa), and -C(368-474aa) were generated by PCR and subcloned into pFLAG-CMV2. Full-length *Drosophila* Numb (556 aa) was subcloned into a pcDNA3 or a pEGFP vectors. Numb and various fragments of Numb, eg., Nb-N (residues 1-76), Nb-PTB (residues 58-205), Nb-C (residues), were cloned respectively into a pGEX4T2 (Amersham-Pharmacia) vector and expressed as GST-fusion in *E.coli*.

A rabbit polyclonal antibody against *Drosophila* Numb were raised using a peptide containing the C-terminal 16 residues of Numb. Anti-NIP antibody was generated similarly using a peptide (CLPNPPVERIRDMHDW) derived from the protein’s C-terminus. Where
necessary, these antibodies were affinity purified and labeled with either FITC or NHS-rhodamine (Pierce). Texas red- and FITC-conjugated anti-rabbit secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. Anti-digoxigenin-AP was from Roche, propidium iodide from Sigma, and TO-PRO-3 from Molecular Probes. A mouse anti-FLAG monoclonal antibody was purchased from Sigma.

**Northern Blot and in situ Hybridization**-Total RNA from the embryonic, larval, pupal or adult stage of *Drosophila* was extracted using the TRZol reagent (Invitrogen, 37). A total of 12.5 µg RNA was separated by electrophoresis on a formaldehyde agarose gel and transferred overnight to a Hybond-N membrane (Amersham Biosciences). The blot was then cross-linked and hybridized at 42 °C to RNA probe (101 bases, corresponding to nucleotides 426-527 of the NIP cDNA) labeled with [α³²P] dCTP. Digoxigenin-labeled RNA probes specific for the full-length NIP and Numb were prepared by *in vitro* transcription with T7 or SP6 RNA polymerase with random priming according to manufacture’s recommendations (Boehringer Mannheim). Embryos were collected, fixed in 4% formaldehyde, hybridized to the probe at 55 °C. After exposing the embryo to anti-Digoxigenin-AP for 2 hours, a color reaction was initiated by incubating with NBT/BCIP.

**Cell Culture, GST Pull-down and Immunoprecipitation**- Human embryonic kidney (HEK) 293 and Cos-7 cells were cultured at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and appropriate antibiotics. For transient transfection, cells were allowed to grow to 50-70% confluence in 10 cm dishes, and ~ 5 µg DNA was added with lipofectAMINE (Life Technologies, Inc.) in serum-free medium. GST pull-down and co-immunoprecipitation experiments were carried out essentially as described (38).
Peptide Synthesis and Fluorescence Polarization—Peptides containing the NERF or the NKGF motif were synthesized on an A431 peptide synthesizer (Applied Biosystems) using standard Fmoc chemistry. Fluorescein-NHS (Molecular Probes) was coupled on-resin to the N-terminus of the peptide in the presence of TEA. The labeled peptide was cleaved off the resin using TFA and purified on HPLC using a Luna C18 column (Phenomenex). Fluorescence polarization measurements were conducted using established procedures as previously reported (38).

Immunofluorescence Microscopy—To observe the localization of NIP in Drosophila, embryos were collected from 3.5 to 4.5 h at 25 °C after egg-laying, dechorionated in 3% bleach, and fixed in 4% formaldehyde in phosphate buffered saline (PBS). The embryos were then blocked for nonspecific reaction using 1% skim milk for 30 min at RT and exposed to the primary antibody for 2 hours. This is followed by a treatment with FITC-conjugated anti-rabbit secondary antibody (used at 1:50 dilution in PBS) for 1 hour at RT. The nuclei of cells were stained with propidium iodide (PI). Confocal images were recorded on a Zeiss confocal microscope (LSM410).

To observe a direct interaction between NIP and Numb in cells, Cos-7 cells were transfected with pEGFP Numb in the presence or absence of pFLAG-NIP or a mutant. Mouse anti-FLAG monoclonal antibody and Texas Red-conjugated anti-mouse secondary antibody (Jackson ImmunoRes. Labs, Inc.) were used to detect NIP in cells. The LSM 410 software was used for image analysis. Reported images were processed using Adobe Photoshop.

RNAi-S2 cells were maintained in Schneider’s Drosophila medium (Invitrogen) with 10% FBS (Sigma) at 23 °C. The RNAi technique was performed according to Clemens et al (39). Full-length NIP (1990bp) single-strand RNA was prepared by in vitro transcription with T7 or SP6 RNA polymerase.
Briefly, cells were diluted to a density of $1 \times 10^6$ cells/ml in Drosophila expression system (DES) serum-free medium (Invitrogen) and plated in 6-well culture dishes with cover slides. Double-stranded (ds) RNA was added directly to the medium. After 1 hour of incubation at RT, 2ml of Schneider’s Drosophila medium with FBS was added and incubated for another 72 hours. For Western blots, cells were harvested and pelleted by centrifugation at 1000 x g. Cell pellets were lysed in 40 µl Laemmli buffer and 20 µl of lysate was used on a 12% SDS-PAGE. Western blot analyses were performed as described above. Anti-NIP and anti-Numb antibodies were used at 1:500 dilution respectively, while anti-β-tublin was applied at 1:5000 dilution.

**RESULTS**

*Isolation of a Drosophila cDNA Encoding a Numb-interacting Protein*- Biotinylated Numb PTB domain was used to screen a 22-24 hr Drosophila embryonic cDNA library. From 500,000 primaries, we obtained four clones of overlapping sequences, representing the partial cDNA of the gene CG4482-PA (40). The EST clone LD14488 (Invitrogen) was used to isolate the full-length cDNA, which encodes a protein of 474 amino acids with a predicted molecular weight of 53,348 daltons (Fig. 1A). The protein was named Numb-interaction protein (NIP). The NIP gene maps to the region 35B7-35B8 on chromosome 2L in the Drosophila genome (40). Sequence analyses by SMART (41, 42) suggest that NIP is a membrane protein with six readily identifiable transmembrane (TM) segments (Fig. 1A). Another potential TM segment located at residues 156-171 (Fig. 1A) has a relatively lower average hydrophobicity compared to those of the other TM segments. When this segment is included, NIP has an overall structure of a 7TM receptor (43). In addition, two candidate Numb PTB domain-binding sites, NERF (residues 134-137) and NKGF (residues 363-366), were found between TM segments 3 and 4 and at the C-terminal
portion of the protein, respectively (Fig. 1A).

Exhaustive homology-based sequence searches against protein databases retrieved several proteins in various species ranging from *Anopheles gambiae* to *Homo Sapiens* that share sequence similarity with *Drosophila* NIP. Significant sequence similarity was detected at the N-terminal halves of the proteins that include the predicted transmembrane segments, while the C-terminal halves were less conserved (Fig. 1B). Of the two candidate Numb PTB-binding sites, the first, represented by a degenerated sequence of F/Y-N-E-x-F-x-W/I, where x represents an undefined amino acid, is conserved in all species examined, whereas the second site, typified by the sequence E-N-K/R-G-F-Q, was shared only by the *Anopheles* and *Drosophila* NIP proteins (Fig. 1B). Interestingly, two mouse NIP (mNIP) variants were identified, of which only one (mNIP2) displays significant sequence identity (70-93%) to the rat (rNIP) or human counterpart (hNIP). All mammalian NIPs, except for hNIP, are truncated at the C-terminus relative to *Drosophila* NIP.

**Identification NIP Transcripts in Whole Drosophila Embryos and the NIP Protein in Embryonic Lysate** - The accumulation of *NIP* transcripts was investigated by Northern blot analysis. Total RNA was extracted from *Drosophila* embryos (0-16 hours), larvae, pupae, and adults, and hybridized to $^{32}$P-labeled *NIP* anti-sense RNA. As shown in Fig. 2A, a transcript of approximately 2.0 kb was detected in embryos, larvae, and adult samples. The size of the transcript corresponds to the length of the cDNA LD14488 (1990 bp). The *NIP* transcript was most abundant in embryos, adults and pupae, and was barely detectable in larvae. This pattern of *NIP* transcript accumulation, particularly the low level observed at the larvae stage, is reminiscent of that of *Numb* (9).
Polyclonal antibodies to the NIP protein were generated using a synthetic peptide corresponding to the C-terminal 16 residues of the predicted protein sequence. A Western blot of Drosophila embryonic lysate revealed a band at 53 kDa, which agrees with the expected molecular weight of 53,348 daltons.

Characterization of a Numb-NIP Interaction in vitro and in vivo- GST pull-down experiments were carried out to examine whether Numb and NIP can interact with each other in vitro. Specifically, GST-NIP immobilized on glutathione beads was used to precipitate Numb from lysate of human embryonic kidney (HEK) 293 cells transfected with Numb cDNA. As seen in Fig. 3A, GST-NIP was capable of bringing down Numb from cell lysate, whereas the control GST failed to do so. In a reciprocal experiment, GST-Numb was found to precipitate NIP from 293 cells (Fig. 3B). These results indicate that NIP and Numb interact specifically with each other in vitro.

To ascertain that this interaction occurs in vivo, we used the Drosophila lysate in a co-immunoprecipitation (co-IP) assay. However, due to the similar size of NIP and immunoglobulin heavy chain, it proved difficult to obtain conclusive results. An alternative strategy was therefore taken using FLAG-tagged NIP. Specifically, 293 cells were co-transfected with expressing constructs for Numb and FLAG-NIP, and the cell lysate was subjected to immunoprecipitation using either a mouse anti-FLAG antibody, a rabbit anti-Numb antibody or non-specific IgG. As shown in Fig. 3C, Numb was detected in anti-FLAG (NIP) immunoprecipitates, but not in those using control IgG. Conversely, NIP was observed to co-IP with Numb (Fig. 3D). These data demonstrate that a Numb-NIP interaction can occur in the physiological setting of a cell.
A PTB Domain-NxxF Motif Interaction Mediates the Association of Numb with NIP-

Although NIP was cloned as a PTB-binding protein, it is necessary to verify whether other regions in Numb contribute to the Numb-NIP interaction. To this end, three truncation constructs corresponding respectively to the N-terminus (Nb-N, residues 1-76), the PTB domain (Nb-PTB, residues 58-205), and the C-terminal region (Nb-C, residues 205-556) of Numb were generated and cloned into a pGEX4T2 vector (Fig. 4A). The Numb fragments expressed as GST fusion were used to bring down NIP from 293 cells. Only Nb-PTB was able to precipitate NIP from 293 cells, whereas the other two fragments of Numb and the control GST failed to do so (Fig. 4B). To determine the regions in NIP responsible for Numb-binding, a series of truncations were made that included none, one, or both of the predicted binding sites for the Numb PTB domain (Fig. 4D). These truncated NIP mutants were expressed in 293 cells, one at a time, in conjunction with full-length Numb. Co-IP experiments were then performed to assess their ability to bind Numb. Only fragments NIP-N1, NIP-N2, and NIP-N1/2, which contain either one or both of the NxxF motifs, were capable of binding Numb. In contrast, the N- and C-terminal fragments (NIP-N and NIP-C), which lack the PTB-binding motifs, did not exhibit appreciable binding (Fig. 4E).

It is clear from these experiments that the PTB domain in Numb and the two NxxF-motifs in NIP are essential for the Numb-NIP protein-protein interaction. To ensure a direct involvement of the NxxF motifs in Numb-binding, two peptides derived respectively from the NERF and the NKGF sites were synthesized and labeled with fluorescein. The affinity of purified Numb PTB domain for each of these peptides was then measured by fluorescence polarization. As shown in Fig. 5A and B, both pep-NERF, representing the first predicted binding site, and pep-NKGF, corresponding to the second binding site, displayed strong binding to the Numb PTB domain with dissociation constants (Kd) of 2.32 and 0.77 µM, respectively, for the corresponding
protein-peptide complexes. These values fall in the same range as for other known Numb PTB domain-peptide complexes (31).

The NERF and NKGF motifs are similar to the NSMF binding motif for Numb in NAK (26). The Asn and Phe residues in the NMSF motif were shown to be essential for the Numb-NAK interaction (31). To determine whether the same residues in the NKGF sequence play an important role in Numb PTB-binding, we performed Ala-scanning substitutions on pep-NKGF using the SPOT technique of multiple peptide synthesis (44). Screening of these peptide analogues for binding to purified Numb PTB domain demonstrated that most residues in pep-NKGF are non-essential for binding as each of them could be replaced by an Ala without compromising binding affinity. However, substitution of the Phe residue by an Ala resulted in a drastic decrease in PTB-binding compared to the original peptide, and a change of Asn to Ala completely abolished binding (Fig. 5B).

To explore the effect of mutations in the NxxF motifs of the NIP protein, we constructed two NIP single mutants, which contain Ala substitutions at either Asn134 (of the NERF site, mutant N1A) or Asn363 (of the NKGF site, mutant N2A), and a double mutant (N1N2/AA) bearing both mutations. Each of the mutants was co-expressed (in FLAG tag) with Numb in 293 cells, and their interaction was assayed by co-IP experiments. Both single mutants, N1A and N2A, retained significant affinities for Numb (Fig. 5C). In contrast, the double mutants N1N2/AA displayed no appreciable binding under the same conditions. These results suggest that the two NxxF motifs in NIP mediate a direct interaction with Numb. Furthermore, the role of these two motifs may be redundant, e.g., the presence of a single motif is sufficient for Numb PTB-binding.

Co-localization of NIP and Numb during Mitosis of Neural Precursor Cells- Given that Numb is an essential protein for asymmetric cell division in the Drosophila nervous system
development and that NIP interacts directly with Numb, it is likely that NIP may play a part in asymmetric cell division. To explore this possibility, embryos at stage 10 were stained with a rabbit anti-NIP antibody to reveal NIP localization in dividing neuroblasts and with propidium iodide (in red) to distinguish the nuclei. NIP was seen to form a crescent at the basal cortex of a neuroblast at prophase, which was maintained through metaphase to telophase (Fig. 6A-D). At telophase, NIP was found predominantly in the small, basal daughter cell (GMC), although a discernable crescent is still maintained on the basal membrane of the apical daughter cell where it contacts the GMC (Fig. 6D). The specificity of the anti-NIP antibody was demonstrated in a negative staining using the secondary antibody alone (Fig. 6K). This pattern of asymmetric localization for NIP during the division of a neuroblast is similar to that of Numb except at prophase (33). To investigate whether these two proteins are colocalized, embryos (stage 10) were co-stained with FITC-conjugated rabbit anti-NIP (green) and rhodamine-labeled anti-Numb (red) antibodies. As shown in Fig. 6 E to J, NIP and Numb exhibited essentially identical staining patterns in neuroblasts during mitosis. Indeed, the two proteins were found to colocalize not only in neuroblasts at metaphase (Fig. 6E-G) but also in the two daughter cells at telophase (Fig. 6H-J). These results indicate that NIP and Numb may be functionally coupled during the asymmetric division of *Drosophila* neuroblasts.

*NIP is both Sufficient and Necessary for the Recruitment of Numb from the Cytosol to the Plasma Membrane* - Since NIP is a membrane protein that interacts specifically and directly with Numb *in vitro* and *in vivo*, we were interested in determining whether NIP can localize Numb to the plasma membrane. We transiently expressed NIP and Numb either singularly or in combination in Cos-7 cells. NIP was found exclusively in the plasma membrane as expected (Fig. 7A). The Numb protein, on the other hand, resided in the cytosol when expressed alone in
Cos-7 cells (Fig. 7B). Co-expression of NIP with Numb in the same cells, however, recruited Numb to the plasma membrane (Fig. 7C-E). The recruitment of Numb was likely through a direct interaction of the two proteins. This idea was confirmed using the N1N2/AA double mutant of NIP. Although the mutant protein localized to the plasma membrane like wild-type NIP, it failed to recruit Numb to the membrane when they were co-expressed in the same cell (Fig. 7F-H). This result demonstrated that the membrane recruitment of Numb was dependent on the NxxF sites of NIP.

To investigate whether NIP is necessary for the membrane association of Numb in *Drosophila* S2 cells, we used an RNA interference strategy to examine the effect of depleting NIP expression on Numb localization. We first established the localization profiles of endogenous NIP and Numb in S2 cells. Both NIP and Numb localized to the plasma membrane of S2 cells (Fig. 7I-K). Treatment of these cells with NIP-specific, double-stranded RNA resulted in silencing of NIP expression in a majority of cells (Fig. 7L). In the cells that lack NIP expression, Numb was found in the cytosol, whereas it stayed on the membrane in the cell that contained NIP (Fig. 7 M & N). It should be noted that, since only a small amount of dsRNA was used, the RNAi-treated cells appeared healthy. The NIP RNAi-induced redistribution of Numb to the cytosol is seen also in Fig. 7O where a cell was co-stained with labeled anti-NIP (green) and anti-Numb (red) antibodies. The RNAi-induced knockdown of NIP was also seen in corresponding Western blots. As shown in Fig. 8, expression of NIP was reduced by approximately 85% in dsRNA-treated cells, while the level of Numb is maintained the same in both treated and control S2 cells. Collectively, these data demonstrate that NIP is necessary for maintaining the membrane association of endogenous Numb in S2 cells.
DISCUSSION

*NIP is a Membrane Anchor for Numb*- A central question in asymmetric cell division is how intrinsic cell-fate determinants are asymmetrically localized during mitosis. Numb, PON, Prospero and Miranda proteins form a basal cortical crescent at prophase when a neuroblast divides along the apical-basal axis and delaminates from the neuroectodermal epithelium (18, 45). Asymmetric localization of determinants is thought to occur in two distinct steps – translocation to the plasma membrane and the formation of distinct cortical crescents (45). Numb does not contain a transmembrane segment or a lipid anchor, and therefore, its recruitment to the plasma membrane may be mediated by a lipid or/and a protein component of the membrane. The N-terminal region of Numb, including the PTB domain, was shown to be necessary for the membrane recruitment and asymmetric localization of Numb (34). Although the PTB domain was originally identified as a protein module that interacts with NPxpY-containing sequences (pY denotes a phosphotyrosine), some members of the family, such as the She and Dab1 PTB domains, are also capable of binding to acidic lipids (46, 47) and are therefore, implicated in membrane localization. However, the Numb PTB domain does not bind specifically to these lipids (Li, data not shown).

The identification of the Numb PTB domain-binding protein NIP provides a plausible mechanism for the membrane localization of Numb. NIP is predicted to be a membrane protein with seven potential TM segments, and is localized to the plasma membrane in Cos-7 cells and in *Drosophila* S2 cells. Numb can be recruited to, and anchored on, the plasma membrane via a direct protein-protein interaction with NIP. Indeed, a direct Numb-NIP protein-protein interaction is mediated by the Numb PTB domain and the two NxxF sites of NIP. In *Drosophila* neuroblasts, NIP colocalizes with Numb in basal cortical crescents. In *Drosophila* embryonic S2
cells, the membrane association of Numb is dependent on NIP, because RNAi-mediated
depletion of NIP protein resulted in Numb relocating to the cytosol. Thus, NIP appears both
necessary and sufficient for the recruitment and retention of Numb in the plasma membrane.
Interestingly, a 76-residue fragment upstream of the PTB domain was localized to the neuroblast
membrane when expressed in Drosophila (34). Since this fragment does not interact with NIP in
vitro, it is likely that it may interact indirectly with NIP or that other mechanisms of Numb
membrane-localization exist.

\textit{NIP and Asymmetric Localization of Determinants-} Does NIP play a role in the formation of
basal Numb crescents in neuroblasts? Although additional work, such as the generation of NIP
mutant flies, is needed to provide a definitive answer to this question, it is tempting to speculate
that NIP may play a part in the asymmetric segregation of Numb. Two lines of evidence support
this idea. First, NIP itself is asymmetrically localized in delaminating neuroblasts and forms a
basal cortical crescent at mitosis. Second, the NIP crescent and the Numb crescent overlay from
prophase through telophase of a cell cycle. It is possible that the formation of a NIP crescent
induced the formation of the Numb crescent by a direct protein-protein interaction.

How is the NIP crescent formed in a neuroblast? The same mechanism that controls the
localization of other basal determinants may also direct the localization of NIP (2). For instance,
Inscuteable is essential for asymmetric Numb localization to the basal cortex in mitotic
neuroblasts (13, 48). Interestingly, Inscuteable itself is localized to the apical membrane, an
event that is dependent on Bazooka, the \textit{Drosophila} homologue of \textit{C. elegans} Par3 (49). In
\textit{Drosophila} neuroblasts and epithelial cells, Bazooka localizes to the apical membrane and forms
a complex with the \textit{Drosophila} Par-6 homologue and the atypical protein kinase C (aPKC)
homologue, DaPKC (50, 51). Bazooka not only provides an apical cue for the correct
localization of Insuteable (49), together with Par-6 and DaPKC, it also directs the localization of basal determinants such as Numb, PON, Miranda, and Prospero (50, 51). In mammalian cells, an analogous complex is formed among mPar3, mPar-6 and aPKC (52-54).

While it is not fully understood how the apical complex of Bazooka/Par-6/DaPKC is anchored to the apical membrane, a possible mechanism is through interactions with membrane proteins such as Crumbs, an integral membrane protein capable of binding to Par-6 through its PDZ domain and thereby recruiting Par-6 to the plasma membrane (54). It is likely that NIP may anchor the basal protein complex of PON, Numb and Miranda through binding to the Numb PTB domain in a fashion akin to the role of Crumbs in the membrane recruitment of Par-6 and the apical complex.

How does an apical complex direct basal segregation of determinants? The missing link between the establishment of cell polarity and asymmetric localization of determinants was recently found in the tumor suppressor, lethal giant larvae (Lgl). Lgl was identified as a substrate of aPKC (55, 56), and phosphorylation of Lgl by the apically localized aPKC results in its inactivation and dissociation from the apical crescent (55, 57). Restricted Lgl activity at the basal crescent appears to be essential for the basal segregation of Miranda and PON (55). It remains to be seen whether basal localization of NIP in a neuroblast is controlled by a similar mechanism.

An Evolutionarily Conserved Role for NIP? The importance of NIP in asymmetric cell division is reflected in the presence of proteins with a high degree of amino acid similarity in other species. Although only one of the two Numb PTB-binding sites identified in Drosophila NIP is conserved in mammalian NIP proteins, our studies indicated that the conserved NExF motif alone was sufficient for mediating a physical interaction between NIP and the Numb PTB domain. This same motif is also capable of mediating an interaction between mammalian Numb
and human NIP (data not shown). It is therefore possible that mammalian NIP proteins play a role in regulating mNumb localization and function.
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REFERENCES


Figure Legends

Figure 1. The nip gene encodes a multi-spanning membrane protein. (A) cDNA sequence of the nip gene and the deduced protein (NIP) sequence. A Kyte-Dolittle (58) hydropathy plot identifies six potential transmembrane (TM) segments (underlined). A potential seventh TM segment is distinguished by italics. The two PTB-binding, NxxF motifs are identified in bold type. (B) Alignment of Drosophila NIP with potential homologues from various other species identified using homology-based sequence search. Conserved residues are color-coded. Red indicates high consensus (greater or equal to 90%) while blue denotes low consensus (equal or less than 50%). An YNExFxW motif shared conserved in all NIP proteins is identified by a rectangle box.

Figure 2. Identification of nip mRNA and NIP protein in Drosophila. (A) Northern blot analysis of NIP mRNA at different developmental stages: embryonic, larval, pupal, and adult. (B) Western blot analysis of NIP protein in lysate of embryos collected from stage 0 through 16.

Figure 3. Characterization of a NIP-Numb interaction. (A) Bacterial expressed and purified GST-NIP was used to pull down Numb from HEK 293 cells while an equal amount of GST protein was used as a control. (B) GST-Numb pulls down NIP from 293 cell lysate. (C) 293 cells co-expressing Numb and FLAG-tagged NIP was subjected to immunoprecipitation by antibodies against either Numb or FLAG or using a non-specific IgG. Western blots were performed using a rabbit anti-Numb antibody. WCL, whole cell lysate, 20% of what was used in co-IP was loaded. (D) NIP was detected in anti-Numb immunoprecipitates. Note that FLAG-NIP co-migrated with the immunoglobulin heavy chain.
Figure 4. Mapping regions in Numb and NIP that mediate their interaction. (A) Constructs of truncated Numb proteins in GST-fusion used in (B). (B) FLAG-NIP expressed in 293 cells were subjected to binding using GST or various GST-Numb truncation mutants. (C) Coomassie-blue staining showing equal application of GST or GST-fusion in B. (D) NIP truncation constructs in a pFLAG-CMV2 vector. N1, N2 denote the NERF and the NKGF sites (shown as rectangles), respectively. (E) Co-immunoprecipitation of Numb and various truncation mutants of NIP (tagged with FLAG) from 293 cells. A monoclonal anti-FLAG antibody was used for immunoprecipitation while a rabbit anti-Numb antibody was employed for Western blot. (F, G) Western blots of cell lysate to demonstrate equal amounts of proteins used in all lanes. Note that a non-specific band at approximately 50 kDa was seen in all lanes of (F).

Figure 5. The Numb PTB domain interacts specifically with the two NxxF motifs in NIP. (A) Binding of peptides pep-NERF and pep-NKGF to the Numb PTB domain. The amino acid sequences are: PDIDYNERFTWEG for pep-NERF, corresponding to residues 129-141 of NIP; and SGGVENKGFQSD for pep-NKGF, corresponding to residues 358-369 of NIP. Each peptide was labeled with fluorescein at the N-terminus. Incremental amounts of purified Numb PTB domain (as GST fusion) were added to the labeled peptide to generate the corresponding binding curve on a Beacon 2000 fluorescence spectrometer (Panvera, CA). No appreciable binding was observed when GST alone was used (not shown). (B) An Ala-scanning SPOT-array of pep-NKGF was screened for binding to purified GST-Numb PTB domain. Sequence of the original peptide is shown above the spots. The first spot represents the original (wild-type) sequence, whereas, subsequent spots represent peptides with one of the amino acids shown
replaced by an Ala. Bright (fluorescent) spots indicate positive binding, while dim or dark spots signal weak or negative binding. (C) Binding of NIP, NIP single mutants N1A and N2A, and a double mutant N1N2/AA to Numb. HEK 293 cells co-expressing Numb and FLAG-NIP or a mutant were subjected to immunoprecipitation using a mouse anti-FLAG antibody. The presence of Numb in the precipitates was verified in a Western blot using a rabbit anti-Numb antibody. The lysate lane contained 20% of the sample used for immunoprecipitation.

**Figure 6. Co-localization of NIP and Numb during mitosis of Drosophila neuroblasts.**

Wild-type embryos at stage 10 were stained using a rabbit anti-NIP antibody and propidium iodide (for DNA) to reveal NIP protein localization at various phases during the mitosis of a neuroblast: (A, B) prophase, (C) metaphase, and (D) telophase. To examine colocalization of NIP and Numb, metaphase neuroblasts were stained with a FITC-conjugated anti-NIP antibody (E). The same cells was co-stained with an anti-Numb antibody labeled with rhodamine (F) to reveal colocalization of NIP and Numb (G). Colocalization of the two proteins was also observed in telophase neuroblasts (H, I, J) where both NIP and Numb were found mainly in the smaller GMC and in the daughter neuroblast as a basal cortical crescent. (K) Negative staining of embryos using FITC-conjugated secondary antibody alone. For clarity, cell boundaries were delineated by broken circles. The apical side is up in all panels.

**Figure 7. NIP recruits Numb to the plasma membrane in vitro and in vivo.** Confocal microscopic images of Cos-7 cells expressing NIP or/and Numb and S2 cells with and without the application of interference dsRNA. Cos-7 cells were made to transiently express either NIP or Numb and were stained for proteins with an antibody against NIP (A) or Numb (B), visualized
by a FITC-conjugated anti-rabbit secondary antibody. DNA is in red. Cos-7 cells co-expressing Numb-GFP and FLAG-NIP (C, D, E) or FLAG-NIP-N1N2/AA double mutant (F, G, H) were stained with a Texas Red-conjugated anti-mouse secondary antibody to reveal its subcellular localization (in red) relative to Numb (in green). Endogenous NIP and Numb proteins in S2 cells were stained respectively with FITC-labeled anti-NIP antibody (green) and rohdamine-labeled anti-Numb antibody (red) (I-K). S2 cells treated with *NIP* dsRNA (L-N) are stained for NIP and Numb as in I-K. An arrow indicates cells that have incorporated the dsRNA and contained no detectable NIP (L) while maintaining normal levels of Numb expression. Note that Numb is localized to the cytosol in the RNAi-treated cells but not in the control cell (M-O).

**Figure 8. Knockdown of NIP expression in S2 cells by RNAi.** Lysate of S2 cells treated or not with NIP-specific dsRNA was subjected to SDS-PAGE and Western blotted respectively using antibodies against NIP, Numb or β-tublin. The level of NIP expression was approximately 15% of that seen in control cells.
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Qin et al. Figure 1B
A

Embryo  Larvae  Pupae  Adult

B

NIP

58

48.5 (kDa)

Qin et al. Figure 2
A

NIP  GST  WCL

Numb

B

Numb  GST  WCL

NIP

C

IP  IgG  □-Numb  IgG  □-FLAG (NIP)

(WB: anti-Numb)

D

IP  □-FLAG (NIP)  □-Numb  WCL

54 H

(kDa)

(WB: anti-FLAG)

Qin et al. Figure 3
Qin et al., Figure 4
A

B

C

IP: FLAG

WB: anti-Numb

Qin et al, Figure 5
Qin et al, Figure 6
dsRNA

-  +

Western blot

Anti-NIP
Anti-Numb
Anti-β-tubulin

Qin et al. Figure 8
A novel transmembrane protein recruits numb to the plasmic membrane in asymmetric cell division
Hanjuan Qin, Anthony Percival-Smith, Chengjun Li, Christina Y.H. Jia, Greg Gloor and Shawn S-C. Li

J. Biol. Chem. published online December 11, 2003

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