LNX was originally cloned as a Numb PTB-binding molecule, and it was subsequently found to act as a RING finger-type E3 ubiquitin ligase for the ubiquitylation and degradation of mNumb. Numb is a PTB domain-containing protein that functions as an intrinsic determinant of cell fate in asymmetric cell division. In mammals, four protein isoforms arise from alternative mRNA splicing. Here we report that while all four protein isoforms bind to LNX, only p72 and p66 Numb isoforms are ubiquitylated and degraded. The p72 and p66 Numb proteins differ from the other two isoforms by the presence of an 11-amino acid sequence insert in the PTB domain (PTBi). We demonstrate that the isoform-specific ubiquitylation of mNumb is due to a novel interaction between the first PDZ domain (PDZ1) of LNX and the PTBi variant. Deletion of LNX PDZ1 domain resulted in loss of ubiquitylation and subsequent degradation of the PTBi form of Numb. Interestingly efficient PTBi ubiquitylation not only depends on association with the LNX PDZ1 domain but also requires binding to the canonical PTB-binding motif NPAY in LNX. Thus two distinct modes of PTBi-mediated interaction with LNX work in concert to allow the effective and specific degradation of the p72 and p66 isoforms of mNumb.

Controlled protein degradation mediated by ubiquitin-dependent proteolysis underlies the regulation of many critical biological events during the life and death of a cell as well as in health and disease (1). Ubiquitylation of a target protein is achieved through a cascade involving at least three enzymes, namely E1, E2, and E3, which activate and transfer ubiquitin to the substrate in a sequential manner. Polyubiquitylated proteins are recognized by the 26 S proteasome and are subsequently degraded into small peptides (2, 3).

E3 ubiquitin ligases are responsible for the specific recognition of a multitude of substrates. Two major classes of E3 have been identified to date characterized by the presence of either a HECT (homologous to E6-AP carboxyl terminus) domain or a RING finger domain. HECT-type E3s catalyze ubiquitylation of a substrate by first forming an E3-ubiquitin thioester intermediate followed by the direct transfer of ubiquitin onto the substrate. On the other hand, RING-type E3s function as scaffolds that bring together the E2 and the target substrate to facilitate efficient transfer of the activated ubiquitin moiety from the E2 to the substrate protein (4). In addition to HECT or RING finger domains, E3 ligases also contain protein interaction modules for substrate recognition. Diversity in the repertoire of E3 substrate selection is achieved by linking a variety of protein interaction modules to the HECT or RING domains. For example, many HECT domain-containing E3s share the WW domain, which is involved in protein-protein interactions and has a role in targeting substrates containing a PY motif for ubiquitylation (5, 6). The RING-type E3 family is composed of two distinct groups, single and multisubunit proteins. Single subunit E3s such as c-Cbl contain the substrate recognition element and the RING finger on the same polypeptide (3). The ability of c-Cbl to recognize tyrosine-phosphorylated receptor protein-tyrosine kinases requires its amino-terminal variant SH2 domain (7). Multisubunit E3s are composed of a complex of proteins including a small RING finger protein and a member of the CULIN family of proteins in addition to other subunits important for functions such as substrate recognition. Among the multisubunit E3s are the anaphase-promoting complex/cyclosome involved in degradation of cell cycle regulators (8, 9), the von Hippel-Lindau-Elongins B and C-Cul2-RING finger complex involved in the degradation of hypoxia-inducible factor 1-α (10), and the Skp1-Cullin/Cdc53-F-box protein-RING finger complexes involved in degradation of signal- and cell cycle-induced phosphorylated proteins (9).

Given the importance of ubiquitin-mediated protein degradation in cellular regulation, high selectivity and specificity of substrate recognition is required. Several distinct modes of substrate recognition by E3 ubiquitin ligases have been described. First, E3s can recognize substrates via their amino-terminal residue known as the N-end rule pathway (11). Second, the binding specificity of protein interaction modules, such as WW domains found in the Nedd4 family of E3s, can dictate substrate selection (12). In addition, substrate phosphorylation can serve as a regulator of E3 recognition and activity. For instance, substrate recognition by F-box proteins in Skp1-Cullin/Cdc53-F-box protein-RING finger complexes involves in degradation of signal- and cell cycle-induced phosphorylated proteins (9).
LNX is a RING-finger-containing E3 ligase first identified as a binding partner for Numb, an adaptor protein implicated in the control of cell fate decisions during development (15, 16). LNX also contains four PDZ domains and interacts directly with the PTB domain of mNumb via the PTB domain-binding motif NPAY (15). We have previously shown that Numb is an LNX substrate and that Numb binding to LNX promotes its ubiquitination and proteasomal degradation. A highly related protein called LNX2 has also been characterized (17) that is capable of binding to the PTB domain of mNumb, although its potential function as an E3 ligase was not tested. In addition to mNumb, the coxsackievirus and adenoovirus receptor was identified as an LNX binding partner (18). The association with coxsackievirus and adenoovirus receptor is mediated by the second PDZ domain of LNX binding to the cytoplasmic tail of receptor. Coxsackievirus and adenoovirus receptor, therefore, may represent a second LNX substrate, although this has not been reported.

While only one form of Numb has been identified in Drosophila, mammals express four alternative spliced isoforms with predicted molecular masses of 65, 66, 71, and 72 kDa. The four Numb protein isoforms differ by the inclusion of two inserts, one of 11 amino acids within the PTB domain and the other of 49 amino acids within the central proline-rich region of the protein. Previously we characterized LNX activity against the p72 Numb isoform that includes both sequence inserts. Since all four isoforms of Numb bind to LNX in vitro, we predicted that all would be susceptible to LNX-mediated ubiquitylation and degradation. However, here we report that only the p72 and p66 Numb isoforms containing the 11-amino acid insert in the PTB domain are ubiquitylated and degraded by LNX. In addition, we show that this selectivity in substrate recognition by LNX requires the first PDZ domain, which mediates a PDZ-PTB domain interaction specific to the variant form of the Numb PTB domain found in p72 and p66.

EXPERIMENTAL PROCEDURES
Plasmids Constructs and Mutagenesis—Wild-type and truncation mutant LNX constructs have been described previously (15, 16). LNX ΔPDZ1 was constructed by ligation of two PCR-generated cDNA fragments containing nucleotides 1–984 and 1296–2372 and subcloning into either pGEX vector (Amersham Biosciences) or pFLAG-CMV2 mammalian expression vector (Sigma). His-tagged PDZ1 of LNX was generated by PCR encoding amino acids 216–446 and then subcloned into pET28a (+) vector (Novagen). GST fusion proteins were constructed by subcloning PCR products into pGEX-4T-3 vectors (Amersham Bioscience). GST-PDZ1 of LNX encodes amino acids 216–446. GST-PDZ2 of LNX encodes amino acids 365–478. GST-PDZ3 of LNX encodes amino acids 495–606. GST fusion protein of the third PDZ domain of PSD95 encodes amino acids 320–427. The cDNAs for the Numb isoforms were subcloned into either pcDNA3.1 (Invitrogen) or pFLAG-CMV2 vector (Sigma). Point mutations were introduced into the Numb using PCR-based mutagenesis. pcDNA-Numb PTBi or PTBo were generated by PCR and encode amino acids 1–246 and 1–325, respectively. GST-PTBi and GST-PTBo have been described previously (15). All cDNA expression constructs and mutations generated by PCR were verified by sequencing.

Cell Culture and Transfection—HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium (Wisent) plus 10% fetal bovine serum. DNA constructs (1–2 µg) were transfected into 293T cells using Lipofectamine (Invitrogen) according to the manufacturer’s instructions.

In Vitro Ubiquitylation Assay—[35S]Methionine-labeled Numb was synthesized in vitro transcription-translation in wheat germ lysates using the TNT kit (Promega). Aliquots (5 µl) of in vitro translated proteins were added into each in vitro ubiquitylation reaction as described previously (16). Briefly in vitro ubiquitylation assays were carried out using 0.6–1.0 µg of purified GST-LNX fusion proteins, 2 µl of E2-containing lysate, 500 mM E1 from yeast (Affinity UW 8545), 2 mM ATP, 5 µM ubiquitin (Sigma), 50 mM Tris-HCl, pH 7.4, 2.5 mM MgCl2, and 0.5 mM dithiothreitol. The reaction mixture was incubated at room temperature for 90 min and stopped by adding 2× SDS sample buffer.

The E2 used was prepared from Escherichia coli BL21 transformed with histidine-tagged Ubc5B DNA in pT7-7 following induction with 1 mM isopropyl-1-thio-galactopyranoside. The bacteria were lysed by sonication in phosphate-buffered saline (PBS) plus protease inhibitors, and the clarified supernatant was incubated for 1 h at 4 °C with nickel-nitritriacetic acid-agarose (Qiagen), which was then washed three times with PBS and was aliquoted and stored at −20 °C.

Immunoprecipitation and Western Blotting—For immunoprecipitation, cell lysates (~1–3 mg of protein) were incubated with appropriate antibody and HNTG-ZE lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 100 mM glyceral, 100 mM ZnCl2, 2 mM EDTA, and protease inhibitors) at 4 °C for 2–4 h. The immune complex was bound to protein A-Sepharose beads, washed three times with cold HNTG-ZE, and eluted by boiling in SDS sample buffer. The immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membranes. For immunoblotting experiments, 50-µg samples of total cellular lysate in SDS loading buffer were separated by SDS-PAGE. Western blot analysis was performed using the enhanced chemiluminescence system (ECL, Amersham Biosciences). Anti-Numb and anti-LNX antibody polyclonal antisera were generated and affinity-purified as described previously (15, 19). Anti-FLAG M2 monoclonal antibody (Sigma) and anti-tubulin (Upstate Biotechnology, Inc.) were purchased.

In Vitro Binding Assays—Numb PTBi and PTBo expression constructs were individually transcribed and translated in the presence of [35S]methionine using wheat germ lysates (TNT kit, Promega). pGEX-LNX constructs were expressed in the BL21 strain of E. coli. Expression of the GST fusion protein was induced with 0.2 mM isopropyl-1-thio-galactopyranoside at 37 °C for 2–4 h. The bacterial pellets were sonicated in PBS plus protease inhibitors. The cleared bacterial lysates were incubated for overnight at 4 °C with glutathione-Sepharose beads (Amersham Biosciences), which were then washed four times with PBS. For the in vitro binding assay, radioabeled Numb PTBi or PTBo was incubated with purified GST fusion LNX proteins on glutathione-Sepharose beads for 2 h at 4 °C in PBS with mixing. Following the incubation, the glutathione-Sepharose beads were pelleted by centrifugation and washed three times in PBS. After washing, pelleted beads were resuspended in 30 µl of SDS sample loading buffer and boiled for 5 min. Unbound proteins were washed into the supernatant were resolved by SDS-PAGE and visualized by autoradiography.

Fluorescence Polarization Measurements—The PDZ1 domain tagged with His6 was expressed in bacteria and purified using nickel-nitritriacetic acid-agarose (Qiagen). Fluorescein labeling and polarization measurements were performed as described previously (37). Equilibrium binding isotherms were obtained by titrating a fixed concentration of fluorescent PDZ1 with an increasing amount of purified PTBi or PTBo protein (as GST fusions). The curves were fit by non-linear regression using Prism curve-fitting software (GraphPad Software, San Diego, CA), which also gave the corresponding dissociation constant (Kd) values. GST alone did not show appreciable binding under the same conditions.

RESULTS
LNX Promotes Numb p72 and p66 Ubiquitylation and Degradation—We have previously shown that LNX interacts with all four isoforms of mNumb through its PTB domain-binding motif, NPAY (Fig. 1A) (19). Therefore, LNX could presumably mediate ubiquitylation of all four Numb isoforms through this interaction. To test this hypothesis, we performed an in vitro ubiquitination assay. Numb p72 was transcribed and translated in vitro in the presence of [35S]methionine and then incubated with recombinant E1, E2 (Ubc5B), and ubiquitin in the presence of GST-LNX fusion protein as E3. The ubiquitinated Numb was shown as a high molecular weight smear (Fig. 1B). To further confirm that the high molecular weight smear is caused by the conjugation of polyubiquitin to Numb, we substituted the untagged ubiquitin (8 kDa) with amino-terminal histidine-tagged ubiquitin, which is 10 kDa, in the in vitro ubiquitination assay. In the presence of His-ubiquitin, the modified Numb proteins were shifted to a higher molecular weight in comparison with that of untagged ubiquitin (Fig. 1B, compare lanes 1 and 2) indicating that the high molecular weights of Numb proteins detected in this assay are indeed modified by ubiquitin. Moreover, in the absence of either E2, ubiquitin, or GST-LNX, Numb p72 failed to be ubiquitinated in vitro (Fig.
Fig. 1. LNX mediated ubiquitylation and degradation of p72 and p66 mNumb isoforms. A, schematic representation of the four mNumb isoforms showing the presence or absence of the PTB insert (gray box in the PTB domain) and the proline-rich region (PRR) insert (hatched box). B, mNumb p72 is ubiquitylated by LNX in vitro. Numb p72 was transcribed and translated in vitro in the presence of [35S]methionine using wheat germ extract and then added to an in vitro ubiquitylation reaction containing E1, E2 (UbcH5B), ubiquitin, and GST-LNX. The reaction of lane 1 contains untagged ubiquitin. Lane 2 contains His-tagged ubiquitin. Lanes 3–5 represent control reactions in which the indicated components in the reaction mixture have been omitted. Reactions were separated by SDS-PAGE, and the mNumb proteins were visualized by autoradiography. An arrowhead indicates the position of unmodified Numb, and the bracket indicates ubiquitylated Numb. C, mNumb p72 and p66 are ubiquitylated by LNX in vitro. All four isoform of mNumb were transcribed and translated in vitro in the presence of [35S]methionine and then added to an in vitro ubiquitylation reaction as described in A. D, LNX-mediated isoform specific ubiquitylation results in isoform-specific degradation of Numb in vivo. 293T cells were transiently co-transfected with all four isoforms of Numb and pFLAG-tagged LNX. 24 h after transfection, whole cell lysates were prepared and quantitated, and equivalent amounts of protein were resolved by SDS-PAGE and immunoblotted with anti-Numb antiserum (top panel). The membrane was then stripped and immunoblotted with anti-LNX to verify LNX expression (middle panel) and anti-tubulin to verify equivalent loading (lower panel). E, to determine whether endogenous p72 and p66 isoforms of Numb were degraded, 293T cells were transiently transfected with the indicated amount of pFLAG-tagged LNX. Equivalent amounts of protein lysate were immunoprecipitated (IP) with either anti-Numb PTBi antibody that specifically recognizes the PTBi form of Numb (top) or anti-Numb antibody that recognize all four isoforms of the Numb protein (bottom) and immunoblotted with anti-Numb serum. aa, amino acids; Ub, ubiquitin; IB, immunoblot; Nb, Numb; NbC, Numb-carboxyl terminus.

These results demonstrate that LNX-dependent Numb ubiquitylation can be detected efficiently and specifically in an in vitro ubiquitylation assay. We then used this assay to examine whether all four isoforms of Numb are ubiquitylated by LNX in vitro. As shown in Fig. 1C, only two isoforms of Numb, p72 and p66, containing the 11-amino acid sequence insert in the PTB domain (PTBi), were efficiently ubiquitylated by LNX in vitro. Since LNX-mediated Numb ubiquitylation further promotes its degradation, we then tested whether LNX-mediated Numb degradation is also isoform-specific. Cells were co-transfected with LNX and each of the four isoforms of Numb, and cell lysates were separated and immunoblotted with anti-Numb antibody. As shown in Fig. 1D, the p72 and p66 Numb isoforms were significantly degraded upon co-expression with LNX, whereas levels of the p71 and p65 isoforms remained relatively unaffected (Fig. 1D). To further investigate whether endogenous Numb degradation is isoform-specific, 293T cells were transfected with LNX, and 24 h later endogenous p72 and p66 numb were immunoprecipitated from cell lysates with an isoform-specific antibody raised against the PTBi insert sequence (19). As shown in Fig. 1E, a dose-dependent degradation of endogenous PTBi containing isoforms of
Numb was observed. In contrast, no appreciable degradation was detected when endogenous Numb was immunoprecipitated with anti-Numb antibody that recognizes all four isoforms (Fig. 1E, lower panel).

**Numb PTBi Domain Is Sufficient for Ubiquitylation by LNX**—Since ubiquitylation of full-length Numb isoforms was dependent on the presence of the insert in the PTB domain, we considered whether the PTBi domain alone is sufficient for ubiquitylation. To address this, two truncation mutants of Numb lacking all amino acids carboxyl-terminal to the PTBi or PTBo domain were generated. The 25 residues at the amino terminus of Numb immediately preceding the PTB domain were retained. The PTBi and PTBo proteins were in vitro translated and used in LNX-dependent ubiquitylation assays. LNX catalyzed ubiquitylation of Numb PTBi but not PTBo in vitro (Fig. 2A).

Typically a polyubiquitin chain is linked to the ε-amino group of one or more internal lysine residues of a substrate. Since there are three lysine residues present within the 11-amino acid PTB insert (Lys-70, Lys-73, and Lys-78 in p72 Numb), we tested whether one of these lysine residues might be the site of Numb ubiquitination and thus mediates the PTBi-specific ubiquitination event. Site-directed mutagenesis was used to substitute each of the lysine residues within the PTB insert with arginine, and mutant forms of Numb p72 were in vitro transcribed and translated in the presence of [35S]methionine. In vitro ubiquitination assays showed that mutation of single lysine residues (K70R, K73R, and K78R) or replacement of all three lysine residues (K70R/K73R/K78R) had no effect on LNX-dependent ubiquitination of p72 in vitro (Fig. 2B). The stability of these Numb mutants was further tested in vivo in cells co-transfected with wild-type LNX. These mutants were degraded as efficiently as wild-type p72 Numb (Fig. 2C). To test the ability of these mutants to bind to LNX, K70R, K73R, K78R, or K70R/K73R/K78R forms of Numb were transiently transfected into 293T cells, and total lysates were incubated with the immobilized GST-LNX fusion protein. Proteins that bound to GST-LNX were identified by Western blotting using anti-Numb antibody. As shown on Fig. 2D, all of the Numb mutants still bound to GST-LNX. Together these data suggest that the three lysine residues within the PTB insert are not sites of ubiquitination that direct the PTBi-specific ubiquitination of Numb. Moreover mutation of these three lysines to arginine did not affect the binding of Numb to LNX.

**LNX PDZ1 Domain Specifically Interacts with mNumb PTBi**—Since the lysine residues specific to the PTBi domain did not appear to be the basis for isoform-specific ubiquitination, we examined the interaction of PTBi and PTBo domains with LNX. We have previously shown that LNX contains a canonical PTB domain-binding motif, NPAY, located between the RING finger domain and the first PDZ domain (15). This PTB-binding motif was required for the Numb-LNX association and for Numb ubiquitination; however, the first PDZ domain of LNX (PDZ1) was also required for Numb p72 ubiquitination both in vitro and in vivo (16). To further define the role of LNX PDZ1 in substrate recognition, we examined whether LNX PDZ1 interacts specifically with the Numb PTB domain. The PTBi and PTBo truncated mutants were labeled with [35S]methionine by in vitro translation and incubated with purified GST-PDZ1. GST-PDZ1 bound to PTBi, and the binding was enhanced with increasing input of GST-PDZ1 fusion protein (Fig. 3A). In contrast, only weak binding to the PTBo domain was observed. To further confirm this interaction, we expressed FLAG-tagged PTBi and PTBo in HEK293T cells and incubated transfected cell lysates with purified GST-PDZ1 protein. GST-PDZ1 bound FLAG-tagged PTBi but not PTBo (Fig. 3B), indicating that the LNX PDZ1 domain interacts specifically with the PTBi isoform. To explore whether this interaction is specific for the PDZ1 domain, we generated GST fusion proteins of isolated PDZ2 and PDZ3 domains and incubated them with PTBi-containing 293T cell lysates. GST-PDZ4 was not tested because a stable GST fusion protein could not be produced. Only LNX PDZ1, but not PDZ2, PDZ3, or GST alone, bound to the Numb PTBi domain (Fig. 3C). Based on the primary amino acid sequence of LNX PDZ1, it most closely resembles those of class I PDZ domains (20). To test whether another member of the same class of PDZ domains could interact with Numb PTBi,
we used a GST fusion of the third PDZ domain of PSD-95, which is most similar to LNX PDZ1. No detectable amount of FLAG-tagged PTBi bound the PSD-95 PDZ domain (Fig. 3 C).

Collectively these data suggest that the first PDZ domain of LNX binds specifically and directly to Numb PTBi.

To ascertain whether the PDZ-PTBi interaction can occur in the context of full-length Numb, we overexpressed all four isoforms of Numb (FLAG-tagged) in 293T cells and performed pull-down assays using GST-LNX PDZ1. GST-PDZ1 preferentially associated with the p72 and p66 Numb isoforms that contain the PTBi variant (Fig. 3D). The much weaker binding of PDZ1 to full-length p65 and p71 may represent nonspecific binding or the presence of a cross-reacting protein. These results lend additional support to the notion that LNX PDZ1 domain associates specifically with Numb PTBi domain and that this association directly contributes to the isoform-specific ubiquitylation of Numb.

To gain a quantitative measure of the interaction between LNX and the Numb PTBi and PTBo variants, we synthesized a peptide corresponding to the NPAY site in LNX and labeled it with fluorescein. Binding of PTBi and PTBo (as GST fusion) to the peptide was measured using fluorescence polarization. As shown in Fig. 4A, both variants were capable of binding to the NPAY peptide with corresponding dissociation constants ($K_d$) of $7.4 \mu M$ for the PTBo and $5.9 \mu M$ for the PTBi domain. Interestingly these $K_d$ values are significantly greater than that obtained for the Drosophila PTB domain binding to similar peptide, which measured $2.4 \mu M$ (data not shown). The contribution of the LNX PDZ1 domain to Numb binding was analyzed using bacterial expressed, purified, and fluorescein-labeled PDZ1. Titration of the PTBi domain protein into fluorescein-PDZ1 produced a saturable binding curve indicative of specific binding between the two proteins (Fig. 4). The corresponding $K_d$ value for this interaction was estimated to be $12.5 \mu M$. In contrast, the PTBo protein exhibited significantly weaker binding ($K_d > 50 \mu M$) under the same conditions (Fig. 4). Collectively these data suggest that while both PTB variants can bind to the NPAY site, only the PTBi domain interacts specifically with the PDZ1 domain of LNX.
Substitution of Lysine Residues within the 11-amino Acid Insert to Alanine Reduced Binding of PTBi Domain to LNX PDZ1—Because the only difference between the PTBi and PTBo variant is an 11-amino acid insert, it seems reasonable to assume that residues within this insert contribute to the preferential binding of PTBi to PDZ1. To explore this possibility, each of the 11 residues, with the exception of the two glycines (Gly-74 and Gly-77), which are not expected to directly participate in specific protein-protein interactions due to the lack of a side chain, was substituted for alanine. These PTBi mutants were subsequently expressed in 293T cells and subjected to pull-down experiments by GST-PDZ1. The PTBi mutants K70A, K73A, and K78A, exhibited significantly reduced binding to the PDZ1 protein as compared with wild-type PTBi, suggesting that these residues play important roles in the Numb PTBi-LNX PDZ1 interaction (Fig. 5A). This assertion was confirmed using PTBi mutants bearing double Lys to Ala substitutions. The binding of the double mutants K70A/K78A and K73A/K78A to PTBi were significantly decreased, whereas that of mutant K70A/K73A was completely abolished (Fig. 5A). These results indicate that residues Lys-78, Lys-73, and Lys-70 in the PTBi insert are important for binding to PDZ1 of LNX. This association may require the presence of several positively charged residues rather than a linear sequence motif since the PTBi domain mutants containing Arg substitutions at these positions still effectively bound LNX and were ubiquitinated as shown in Fig. 2. Furthermore a peptide corresponding to the PTBi sequence was unable to compete for PDZ1 binding (data not shown) indicating that PTBi-PDZ1 binding involves the intact PTBi domain rather than recognition of the insert peptide sequence by PDZ1.

Since mutation of K70A/K73A severely attenuated the binding of Numb PTBi to LNX PDZ1 we tested whether this mutant in full-length p72 Numb would also abrogate its ubiquitylation. To address this question, we generated full-length K70A/K73A, K70A/K78A, and K73A/K78A mutant forms of p72 and then performed in vitro ubiquitylation assays. As shown in Fig. 5B, the K70A/K73A mutation dramatically decreased the level of Numb ubiquitylation as compared with wild-type p72, whereas K70A/K78A and K73A/K78A showed a level of ubiquitylation similar to that of wild-type p72. However, we did observe some modified forms of K70A/K73A that are not present in the absence of LNX suggesting that this mutant still serves as a weak substrate for LNX. We next examined the stability of these mutants in vivo. Mutant forms of p72 were transiently transfected into 293T cells together with either pFLAG-CMV empty vector or FLAG-tagged LNX. In agreement with the results from the in vitro ubiquitylation assay, K70A/K73A was resistant to LNX-dependent degradation in vitro, whereas overexpressed K70A/K78A and K73A/K78A were degraded as efficiently as wild-type p72 (Fig. 5C). Taken together, these results suggest that Lys-70 and Lys-73 play a key role in mediating the interaction between PTBi and PDZ1 of LNX and that the PTB-PDZ interaction is important for the efficient ubiquitylation and degradation of the PTBi form of Numb. The Interaction between LNX PDZ1 and Numb PTBi Is Required for PTBi-specific Numb Ubiquitylation and Degradation—The presence of two interaction sites for PTBi as compared with one for PTBo suggests that the former may form a more stable complex with LNX, and this difference in binding may directly underlie PTBi-specific Numb ubiquitylation. To test this assertion, PTBi and PTBo were radioactively labeled by in vitro translation in the presence of [35S]methionine and subsequently incubated with LNX or various truncation mutants of LNX (Fig. 6A). Truncated LNX containing the NPAY motif but not the PDZ domains (LNX-ΔPDZ) bound to PTBi and PTBo with similar strength (Fig. 6B) in agreement with the comparable affinities determined for these two PTB variants toward the NPAY-containing peptide. The importance of the NPAY motif in PTB binding was demonstrated using a mutant, LNX-ΔPDZ(188A), in which the Tyr residue within the motif was replaced by an Ala. As seen in Fig. 6B (lower panel), this mutant was unable to bind to either PTBi or PTBo.

Inclusion of the PDZ1 domain (LNX-ΔPDZ234) led to a significant enhancement in binding to PTBi but had little effect on binding to PTBo (Fig. 6B). Similarly full-length LNX displayed
PTBi ubiquitylation. Isolated Numb PTBi domain was transcribed and translated washing, radioactive protein retained was visualized by SDS-PAGE followed by autoradiography. Isolated Numb PTBi domain was transfected and translated \textit{in vitro} with wild-type (WT) or mutant LNX. Ubiquitylation of Numb PTBi was visualized by autoradiography. D, HEK293T cells were transiently co-transfected with p72 Numb and either FLAG-CMV\_2 vector, FLAG-tagged LNX\_WT, LNX\_ΔPDZ1, LNX\_Y188A, or LNX\_C48A. 24 h post-transfection, whole cell lysates were prepared, and equivalent amounts of protein were resolved by SDS-PAGE and immunoblotted with anti-Numb (top), anti-LNX (middle), or anti-tubulin (bottom) antibody. WT, wild type; Ub, ubiquitin; IB, immunoblot.

Fig. 6. A, schematic representation of wild-type LNX and varied LNX mutants. B, the PDZ1-PTBi interaction increased the binding of LNX to Numb PTBi but not to PTBo. The indicated GST-LNX fusion proteins were incubated with \textsuperscript{35}S-labeled PTBi or PTBo proteins. After extensive washing, radioactive protein retained was visualized by SDS-PAGE followed by autoradiography. C, deletion of PDZ1 of LNX caused a loss of Numb PTBi ubiquitylation. Isolated Numb PTBi domain was transcribed and translated \textit{in vitro} in the presence of \textsuperscript{35}S-methionine and then added to the \textit{in vitro} ubiquitylation assay in the presence of wild-type or mutant LNX. Ubiquitylation of Numb PTBi was visualized by autoradiography. D, HEK293T cells were transiently co-transfected with p72 Numb and either FLAG-CMV\_2 vector, FLAG-tagged LNX\_WT, LNX\_ΔPDZ1, LNX\_Y188A, or LNX\_C48A. 24 h post-transfection, whole cell lysates were prepared, and equivalent amounts of protein were resolved by SDS-PAGE and immunoblotted with anti-Numb (top), anti-LNX (middle), or anti-tubulin (bottom) antibody. WT, wild type; Ub, ubiquitin; IB, immunoblot.

Thus, a substantial greater affinity for PTBi than for PTBo. Moreover, disruption of PTB-binding motif in full-length LNX (LNX\_Y188A) abrogated LNX binding to PTBo, while binding to PTBi, at a level comparable with that observed using the PDZ1 domain alone, was retained (Fig. 6B). Together these observations reinforce the notion that the PDZ1 domain represents a second interaction surface in LNX for the PTBi but not the PTBo domain.

To explore the physiological relevance of the PDZ1-PTBi interaction, we generated a GST-LNX mutant that specifically deletes the PDZ1 domain (GST-ΔPDZ1) and subjected it to an \textit{in vitro} ubiquitylation assay. GST-ΔPDZ1 was not able to catalyze PTBi ubiquitylation (Fig. 6C). As we have previously shown (16), LNX-ΔPDZ234 that contains both the NPAY motif and PDZ1 domain was capable of catalyzing PTBi ubiquitylation as efficiently as wild-type LNX. Disruption of the NPAY motif, as in mutant LNX\_Y188A, led to failure in polyubiquitylation of Numb PTBi (Fig. 6C). Moreover LNX-ΔPDZ with the NPAY motif but no PDZ domains was also unable to ubiquitylate Numb PTBi. Together these data indicate that PTBi binding to both the NPAY motif and PDZ1 is required for effective recognition of Numb as a substrate and that neither mode of binding on its own is sufficient for Numb ubiquitination. We further tested whether LNX\_Y188A and LNX-ΔPDZ1 affected the degradation of Numb p72 \textit{in vivo}. Compared with wild-type LNX, neither LNX\_Y188A nor LNX-ΔPDZ1 was capable of promoting degradation of overexpressed p72 Numb in 293T cells (Fig. 6D).

DISCUSSION

Cell fate determinants such as Numb function by being selectively segregated into, or excluded from, one daughter cell during cell division. Consequently the daughter cell that receives Numb adopts a distinct identity (21–25). In \textit{Drosophila} embryos, overexpression or deletion of Numb produces opposite phenotypes (26), reflecting a switch in cell fate specification. Similarly overexpression of avian Numb in the neuroepithelium promotes neurogenesis (27), and loss of Numb in the developing nervous system of the mouse embryos impairs the ability of progenitor cells to undergo asymmetric division producing a neuronal daughter and another progenitor cell (28). Since the presence of Numb protein in a cell influences its identity, understanding how the cellular levels of Numb are regulated is important to the elucidation of its function in asymmetric cell division. Previously we have shown that the intracellular level of mNumb is regulated by LNX, an E3 ligase that mediates its ubiquitylation-promoted degradation. In the present study, we have found that LNX specifically targets the p72 and p66 isoforms of mNumb for ubiquitylation, and this selectivity was conferred by a novel interaction between the PDZ1 domain of LNX and the PTBi domain of mNumb.

The PTBi domain of mNumb is characterized by an 11-amino acid insert that is enriched in Lys residues. \textit{In vitro} binding studies using PTBi mutants indicate that these Lys residues play an important role in mediating binding to the PDZ1 domain. Although the PTB-PDZ interaction is necessary for the \textit{in vitro} ubiquitylation of PTBi and for isoform-specific mNumb ubiquitylation and degradation \textit{in vivo}, it is not sufficient for these events to occur. Indeed a second canonical PTB-binding motif in LNX, NPAY, is needed for effective ubiquitylation of both PTBi and PTBi-containing mNumb isoforms. In contrast, the PTBo domain from the p71 and p65 isoforms of mNumb, which lack the 11-residue insert, failed to bind the PDZ1 do-
main of LNX, although its binding affinity for the NPAY site is similar to that of the PTBi variant. This difference apparently accounts for the inability of LNX to ubiquitylate either the PTBo domain or the PTBo-containing mNumb isoforms.

A Novel PDZ-PTBi Interaction—A direct physical interaction was observed between the LNX PDZ1 domain and the mNumb PTBi domain variant. To our knowledge, this is the first example of an interaction between these two types of protein modules. What is the molecular basis underlying such an interaction? The Numb PTB domain is flexible in terms of binding specificity and is capable of binding multiple proteins, including PON, Nak, LNX, Mdm2, and Siah (18, 29–31). Structural analysis of the Drosophila Numb PTB domain revealed that it possesses a large hydrophobic binding surface, allowing the recognition of different ligands through the use of either a part of, or the entire, binding surface (32). Moreover the PTB domain has the same general fold as a pleckstrin homology domain, another protein module capable of interacting with both lipids and proteins (33). In fact, some members of the PTB domain family such as Shc can bind phospholipid and phosphotyrosine-containing ligands simultaneously by engaging two different parts of the PTB domain surface (34). We have shown previously that the PTB domain of mammalian Numb is also capable of binding phospholipids (19).

This flexible binding is not confined to PTB domains. For example, the SH2 domain protein SAP binds simultaneously to both the Fyn SH3 domain and a Tyr phosphorylation motif in the T-cell co-receptor SLAM using two distinct sites, and in so doing, it serves a role of an adaptor for Fyn and SLAM (35–37). The Numb PTB domain may bind to both the NPAY motif and the PDZ1 domain in an analogous manner. Although a structure of a PTB-PDZ1 complex would provide a definitive answer for the molecular basis of such a novel interaction, structure-based sequence alignment of mNumb PTB1 domain against that of the Drosophila PTB domain (38) places the 11-residue insert between the α-helix A2 and strand B2 predicted to be a distinct surface from the predicted binding site for the NPAY motif. Because this short insert is the only difference in sequence between the PTBi and PTBo variants, it is reasonable to assume that this insert is involved in binding the PDZ1 domain either directly or indirectly through conformational modifications of the PTBi domain. In support of such a possibility, substitutions of insert residues Lys-70, Lys-73, or Lys-78 with alanine resulted in a significant decrease of binding affinity for PDZ1. A peptide corresponding to the insert failed to compete for binding to PDZ1 (data not shown), implying that the PTB insert alone does not represent a linear binding motif. We cannot exclude the possibility, however, that the isolated insert peptide in solution may not adopt the correct conformation necessary for binding.

Another interesting possibility is that the PTBi domain may form a complex with PDZ1 in a manner akin to the dimerization of two PDZ domains. Although most PDZ domains promote protein-protein interactions through the recognition of peptide motifs at the carboxyl terminus of a protein, which usually ends with a hydrophobic residue (39, 40), some PDZ domains are capable of forming homo- or heterodimers (41, 42). The dimerization of PDZ domains is based on the recognition of internal peptides. Structural studies have revealed that in specific cases the internal peptide adopts a β-hairpin finger structure, and this β-finger docks in the peptide-binding groove, mimicking a peptide-ligand interaction (43). Since the PTB and PDZ domains share a common structural fold characterized by an anti-parallel β-sheet (44), the same mode of interaction adopted by PDZ-PDZ dimerization may be utilized by the mNumb PTBi-LNX PDZ1 complex.

The Role of the PDZ-PTBi Interaction in Numb Ubiquitylation—How does the PDZ-PTBi interaction contribute to LNX-dependent, isorm-specific Numb ubiquitylation? Two scenarios can be envisaged. First, binding of a PDZ domain to the PTBi domain could confer high selectivity and specificity in substrate recognition by LNX. Recognition of a substrate by an E3 ligase is generally achieved by a specific and regulated interaction. Nevertheless accumulating evidence suggests that not all proteins that can interact with an E3 are physiological substrates (45). This is consistent with our finding that not all four isoforms of mNumb are substrates of LNX despite being able to interact with LNX via the canonical PTB-binding motif NPAY. To achieve efficient ubiquitylation, an additional interaction between PDZ1 and PTBi is required. The PTBi domain displayed relatively weak binding affinities for both the NPAY peptide and the PDZ1 domain in fluorescence polarization measurements. While the latter affinity might have been underestimated due to modifications of the protein surface as a result of fluorescein labeling, the two binding sites could synergize with each other to confer a stronger and physiologically significant interaction between LNX and Numb PTBi, leading to effective ubiquitylation of the latter. In comparison, the PTBo domain, which lacks binding at the two sites, is not efficiently ubiquitylated by LNX.

The selective ubiquitylation of PTBi-containing Numb isoforms, therefore, suggests that there may be a threshold for the E3 ligase activity of LNX and that this threshold is fine tuned by the affinity of substrate binding. The Numb PTBo domain contains a binding site for LNX; however, the interaction mediated by such a site may be below the threshold for LNX activation. In contrast, PDZ1 binding, together with an NPAY motif-mediated interaction, may increase the binding affinity above such a threshold. This type of interaction, in which multiple low affinity interactions set the threshold for substrate selection, was shown to regulate the interaction between the F-box protein Cdc4 and the cyclin-dependent kinase inhibitor Sic1 (46). Targeting of the phosphorylated form of Sic1 for ubiquitylation by Cdc4 requires multisite phosphorylation of six of the nine phosphorylation sites of Sic1 to achieve high affinity binding (46, 47).

The second possible route by which the PDZ1-PTBi interaction may regulate LNX function is by regulating its catalytic activity. It is believed that RING finger-type E3s catalyze ubiquitylation through a mechanism that involves the recruitment and productive positioning of the substrate relative to the E2 active site. This model is based on the structures of the Cul-Rbx1-Skp1-F-boxSkp2 complex (48), of the leucine-rich repeat-containing F-box protein Skp2 bound to Skp1 (49), and of the RING E3 c-Chl bound to an E2 (50). The interaction between PDZ1 and the PTBi may in fact orient Numb in a manner such that it is optimally presented to the E2 active site.

LNX-dependent Numb Degradation in Numb Localization—Asymmetric Numb distribution is essential for its function as a cell fate determinant. In Drosophila, this is achieved by the formation of a polarized Numb membrane crescent. By aligning the mitotic spindle perpendicular to such a crescent, Numb is selectively distributed into one of the two daughter cells; however, the molecular and cellular mechanisms by which Numb achieves its asymmetric membrane localization are not well understood. In principle, it can be accomplished by one or a combination of several possible mechanisms: asymmetric localization of mRNA, active transport of the proteins by motor molecules along the cytoplasmic or cortical cytoskeleton, trapping of the protein by asymmetrically localized anchor molecules, or protein targeting to the membrane followed by selective degradation in one region of the cortex (51).
PTB-PDZ Domain Interaction Mediates Ubiquitylation of Numb

It is possible that LNX could contribute to the asymmetric distribution of Numb by promoting the ubiquitylation and degradation of membrane-associated mNumb, in particular the PTB1-containing isoforms, in a similar manner. The presence of multiple PDZ domains in LNX may provide a mechanism for its localization to distinct cell membrane compartments. PDZ domain-containing proteins are often localized to discrete submembranous sites and are found in several distinct cell polarity complexes. It is then, reasonable to speculate that LNX could be specifically localized at certain regions of the cell cortex and thus promotes the localized degradation of Numb so as to achieve its asymmetric distribution of Numb within the cell.

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