LNX functions as a RING type E3 ubiquitin ligase that targets the cell fate determinant Numb for ubiquitin-dependent degradation

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LNX is a RING finger and PDZ domain containing protein that interacts with the cell fate determinant Numb. To investigate the function of LNX, we tested its RING finger domain for ubiquitin ligase activity. The isolated RING finger domain was able to function as an E2-dependent, E3 ubiquitin ligase in vitro and mutation of a conserved cysteine residue within the RING domain abolished its activity, indicating that LNX is the first described PDZ domain-containing member of the E3 ubiquitin ligase family. We have identified Numb as a substrate of LNX E3 activity in vitro and in vivo. In addition to the RING finger, a region of LNX, including the Numb PTB domain-binding site and the first PDZ domain, is required for Numb ubiquitylation. Expression of wild-type but not mutant LNX causes protesome-dependent degradation of Numb and can enhance Notch signalling. These results suggest that the levels of mammalian Numb protein and therefore, by extension, the processes of asymmetric cell division and cell fate determination may be regulated by ubiquitin-dependent proteolysis. 

Keywords: LNX/Numb/PDZ domain/RING finger/ubiquitin ligase

Introduction

The numb gene in Drosophila affects binary cell fate decisions of cells in the peripheral and central nervous system, as well as muscle cells during development (Uemura et al., 1989; Brewster and Bodmer, 1995; Spana and Doe, 1996; Ruiz and Bate, 1997; Park et al., 1998). Drosophila Numb (dNumb) is a membrane-associated protein expressed in progenitor cells of these lineages. During cell division, dNumb asymmetrically localizes and subsequently segregates to one daughter cell, where it functions as an intrinsic determinant of cell fate (Rhyu et al., 1994; Knoblich et al., 1995; Spana et al., 1995; Vervoort et al., 1997). Evidence suggests that, in Drosophila, Numb proteins influence cell fate by inhibiting the action of Notch (Frise et al., 1996; Guo et al., 1996; Spana and Doe, 1996).

Conserved Numb genes have been identified in mouse, rat, chicken and human (Verdi et al., 1996; Zhong et al., 1996; Wakamatsu et al., 1999). In addition, a related gene called Nbl (Numblike) has also been identified in mammals (Zhong et al., 1997; Dho et al., 1998). Heterologous expression of mammalian Numb (mNumb) in Drosophila produces a phenotype (transformation of hair cells to neurons) similar to ectopic expression of dNumb, suggesting that these proteins have conserved functional properties (Verdi et al., 1996; Zhong et al., 1996). In addition, there is evidence that the vertebrate Numb protein is asymmetrically distributed in dividing neuroepithelial cells that undergo asymmetric cell divisions and can inhibit the ability of Notch to suppress neuronal differentiation (Zhong et al., 1996; Wakamatsu et al., 1999). Homozygous deletion of the Numb gene in mice results in early embryonic lethality (Zhong et al., 2000; Zilian et al., 2001). Together, these results suggest that the levels of Numb protein are important in regulating developmental processes controlled by Notch. However, the mechanisms that regulate the asymmetric distribution and the levels of the vertebrate Numb protein are currently unknown.

The structure of both Drosophila and vertebrate Numb proteins suggests that they function as adaptor proteins to mediate the formation of multiprotein complexes. mNumb has an N-terminal phosphotyrosine-binding (PTB) domain that interacts with ligands in a phosphotyrosine-independent manner (Li et al., 1997, 1998; Dho et al., 1998; Yaich et al., 1998). mNumb PTB binding partners include Notch, mdm2 and LNX, although the biological importance of these interactions has not yet been established (Zhong et al., 1996; Dho et al., 1998; Juven-Gershon et al., 1998). Within its C-terminal region, Numb also contains an Eps15 homology (EH) domain-binding motif (Salzini et al., 1997; Paoluzi et al., 1998) and two tripeptide sequences that fit the binding motif for α-adaptin, a component of the AP-2 complex. Mammalian Numb has recently been demonstrated to localize to clathrin-coated pits and early endosomes, and overexpression of fragments of Numb can block internalization of the EGFR and transferrin receptors, implying a role for Numb in intracellular vesicle trafficking events (Santolini et al., 2000).

Previously, LNX was identified as a mNumb PTB domain-interacting protein (Dho et al., 1998). In addition to the Numb PTB domain-binding motif, LNX contains four PDZ domains, which also presumably mediate protein–protein interactions although their targets are not yet identified. LNX also contains an N-terminal RING finger domain. RING finger domains are conserved, cysteine-rich, zinc-binding domains found in a diverse group of proteins that until recently appeared to be functionally unrelated (Borden and Freemont, 1996;
Saurin et al., 1996). However, an accumulating body of evidence suggests that many RING finger-containing proteins are involved in protein ubiquitylation (Tyers and Willems, 1999; Jackson et al., 2000; Joazeiro and Weissman, 2000). The ubiquitylation pathway involves a multiprotein cascade in which the first step is the attachment of free ubiquitin to a ubiquitin-activating enzyme (E1). Ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2), which along with a ubiquitin ligase (E3) protein, ligates the ubiquitin to a specific protein target (Hershko and Ciechanover, 1998). The specificity of this process is determined by the E3 component, which is proposed to function as an adaptor to bind substrates selectively (Tyers and Willems, 1999; Joazeiro and Weissman, 2000). Ubiquitylated proteins are then targeted to the proteasome or, in some cases (as with some plasma membrane proteins), to the endocytic pathway, resulting, in either case, in the degradation of the protein (Hershko and Ciechanover, 1998; Hicke, 1999; Rotin et al., 2000).

RING finger domain proteins Rbx1 or Apc11 function as components of ubiquitin ligase complexes such as Skp1–Cdc53/CUL1-F-box protein (SCF), anaphase-promoting complex (APC) and VHL–elonginC/elonginB (VCB), and facilitate the transfer of ubiquitin from E2 to the substrate (Tyers and Willems, 1999). Another large family of RING finger-containing proteins, such as Cbl, Siah-1 and mdm2, which are otherwise unrelated to Apc11 or Rbx1, also have RING finger-dependent E3 ubiquitin ligase activity (Hu et al., 1997; Joazeiro et al., 1999; Lorick et al., 1999). In light of the mounting evidence for E3-like activity of proteins with RING finger domains, we tested whether LNX could promote ubiquitin-mediated protein degradation. Here we show that LNX has E3 ubiquitin ligase activity and this activity requires an intact RING finger domain. Furthermore, we identify Numb as a LNX substrate and find that expression of LNX results in the ubiquitylation and degradation of Numb proteins.

Results

The LNX RING finger domain mediates E2-dependent ubiquitylation activity

To determine whether LNX can function in a manner similar to the recently characterized RING finger domain-containing E3 ubiquitin ligases, recombinant glutathione S-transferase (GST)–LNX was used in an in vitro ubiquitylation assay. GST–LNX fusion proteins were incubated with ubiquitin, recombinant E1 and E2 (UbcH5B), and ubiquitin ligase activity measured by detection of ubiquitylated GST fusions proteins using anti-ubiquitin antibodies. E2-dependent ubiquitin ligase activity was detected in reactions containing pGST–LNXWT but not in reactions containing a form of LNX in which a conserved cysteine residue (C48) is mutated to alanine (LNXC48A; Figure 1A). The isolated RING finger domain also promoted E2-dependent E3 activity and mutation of C48 abolished this activity (Figure 1A). To further assess whether LNX has E3 activity in vivo, control T cells were co-transfected with HA-tagged ubiquitin together with increasing amounts of FLAG-tagged LNXWT or LNXC48A. Anti-HA immunoblotting revealed a dose-dependent increase in the ubiquitylated proteins detected in LNXWT immunoprecipitates but not LNXC48A (Figure 1B).

LNX promotes the ubiquitylation of Numb

LNX was initially identified as a Numb PTB domain-binding protein, therefore we tested whether Numb is a substrate for LNX-mediated ubiquitylation. The p72 isoform of Numb 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 purified GST or GST–wild-type or C48A LNXC48A fusion proteins. In the presence of wild-type GST–LNX, Numb was modified significantly, as indicated by the shift in migration of the input Numb protein into discrete bands and a high molecular weight smear (Figure 2A). The C48A mutant form of GST–LNX and GST alone did not cause a change in Numb mobility. To test whether LNX would promote Numb ubiquitylation in vivo, FLAG-tagged LNXWT or LNXC48A was co-transfected into 293T cells with HA-tagged ubiquitin.
Endogenous Numb proteins were immunoprecipitated from cell lysates with anti-Numb antibody and immunoblotted with anti-HA antibody to detect ubiquitylated proteins. As shown in Figure 2B, a dose-dependent accumulation of ubiquitylated proteins was detected in Numb immunoprecipitates from LNX WT-transfected cells but not LNX CAAX-transfected cells. To determine whether Numb itself is ubiquitylated, rather than Numb-associated proteins, the cell lysates were boiled in the presence of SDS to disrupt protein–protein interactions and then immunoprecipitated with anti-HA. Numb-reactive bands were detected in anti-HA immunoprecipitates from boiled lysates, indicating that Numb was directly modified by HA-ubiquitin in the presence of wild-type but not mutant LNX (Figure 2C). Furthermore, in the presence of a proteasome inhibitor, MG132, Numb reactivity could be detected as a high molecular weight smear, suggesting that it had been modified by polyubiquitylation.

**LNX substrate recognition requires the PTB domain-binding motif and the first PDZ domain**

Previously, we have shown that the interaction between LNX and Numb requires the NPAY(188) sequence motif between the RING finger domain and the first PDZ domain (Figure 3A), which serves as a phosphorylation-independent binding site for the Numb PTB domain (Dho et al., 1998; Li et al., 1998). To identify regions of LNX important for substrate recognition, we generated a series of truncation mutants, as depicted in Figure 3A. All of the LNX truncation mutants retained E2-dependent ubiquitin ligase activity in vitro, confirming that the RING finger domain is sufficient for E2 activation (Figure 3B). We then tested the ability of the LNX truncation mutants to ubiquitylate Numb in vitro. Wild-type LNX and LNX mutants lacking PDZ domains 2, 3 and 4 efficiently ubiquitylated in vitro translated Numb (Figure 3C). However, removal of the first PDZ domain resulted in a complete loss of substrate recognition, even though LNX-APDZ retains the Numb PTB domain-binding site.

The regions of LNX required for ubiquitylation of endogenous Numb were determined in cells co-transfected with mammalian expression vectors encoding FLAG epitope-tagged LNX or the LNX mutants depicted in Figure 3A and HA epitope-tagged ubiquitin. Anti-Numb immunoprecipitates were immunoblotted with anti-HA monoclonal antibody (top). The membrane was then stripped and reprobed with anti-Numb antibody (middle). Total cell lysates were blotted with anti-LNX to monitor LNX expression levels (bottom, as in Figure 1B). (C) 293T cells were transiently co-transfected with pMT HA-ubiquitin and either pFLAG-LNX WT or LNX CAAX used in Figure 1B and immunoblotted with anti-HA monoclonal antibody (top). The membrane was then stripped and reprobed with anti-Numb antibody (middle). Total cell lysates were blotted with anti-LNX to monitor LNX expression levels (bottom, as in Figure 1B). (C) 293T cells were transiently co-transfected with pMT HA-ubiquitin and either FLAG-CMV-2 vector alone, FLAG-tagged LNX WT or LNX CAAX, which disrupts the RING finger domain. Some transfected cells were incubated overnight with 20 μM MG132 before harvest. Cells were lysed in 1 ml of modified HNTG-ZE lysis buffer containing 1% SDS and then boiled at 95°C for 5 min. The lysates were then diluted with 10 ml of HNTG-ZE lysis buffer and subjected to immunoprecipitation with anti-HA antibody, separated by SDS–PAGE and immunoblotted with anti-Numb antibody.
and fourth PDZ domains (LNXΔ234) appeared to bind to Numb and ubiquitylate substrates more efficiently than wild-type LNX (Figure 4A and C), suggesting that PDZ domains 2, 3 and 4 may have a regulatory function. In addition, some of the ubiquitylated proteins detected in LNXΔ234 transfected cells are likely to represent ubiquitylated forms of LNX, since these high molecular weight bands were also detected by anti-FLAG antibodies (Figure 4C and D).

**LNX expression results in Numb protein degradation**

Ubiquitylation of cellular proteins often leads to their degradation by the proteasome. To determine whether LNX-mediated ubiquitylation of Numb promotes its degradation, 293T cells were co-transfected with wild-type, C48A or Y188A mutant forms of LNX and p72 Numb. Protein lysates of transfected cells were separated and immunoblotted with anti-Numb. Transfected Numb was significantly degraded when co-transfected with wild-type LNX but not when transfected with the LNX mutants that were unable to ubiquitylate Numb efficiently (C48A and Y188A mutants; Figure 5A).

E3 enzymes are largely responsible for target specificity and therefore we tested whether Numb was a specific target of LNX or whether overexpression of c-Cbl, another RING finger domain containing E3, could cause degradation of co-transfected p72 Numb. Numb was co-transfected with LNX or c-Cbl in 293T cells and total protein lysates from transfected cells were immunoblotted with anti-Numb antiserum. In contrast to LNX transfected cells, in which most of the transfected Numb was degraded, transfection with c-Cbl had no detectable effect on p72 Numb protein levels (Figure 5B).

To determine whether Numb degradation in the presence of LNX is proteasome dependent, 293T cells were co-transfected with p72 Numb and wild-type LNX and treated with either 10 or 25 µM MG132 for 6–22 h post-transfection. Numb protein levels were examined by immunoblotting of total cell lysates with anti-Numb antibodies.
Fig. 4. LNX substrate recognition and co-immunoprecipitation with Numb in vivo requires the PTB binding motif and the first PDZ domain. 293T cells were co-transfected with pMT HA-ubiquitin and FLAG-tagged mutants of LNX as depicted in Figure 3A. Cell lysates were prepared 24 h after transfection and Numb immunoprecipitated from 1 mg of protein. Immunoprecipitates were resolved by SDS–PAGE and transferred to PVDF membrane. Replicate membranes were blotted with anti-HA to detect ubiquitylated proteins (A), anti-Numb (B) to detect precipitated Numb proteins and anti-LNX (C) to detect LNX co-immunoprecipitating with Numb. Whole-cell lysates (D) were blotted with anti-LNX antibody to confirm expression of the mutant LNX proteins.

Fig. 5. LNX-mediated ubiquitylation results in degradation of Numb. (A) 293T cells were transiently co-transfected with pcDNA-p72 Numb and either pFLAG-CMV-2 vector alone, FLAG-tagged LNXWT or LNX mutants that disrupt either the RING finger domain (LNXCAK) or the Numb PTB domain binding site (LNXT188A). Twenty hours after transfection, whole-cell lysates were prepared, quantitated and equivalent amounts of protein resolved by SDS–PAGE and immunoblotted with anti-Numb antiserum (top). The membrane was then stripped and immunoblotted with anti-LNX to verify equivalent loading (bottom). (B) To determine whether overexpression of another RING domain containing E3 could promote degradation of Numb, cells were co-transfected with pcDNA-p72 Numb and either empty pFLAG-CMV-2 vector, wild-type FLAG-tagged LNX or HA-Cbl. Total cell lysates were quantitated and equivalent amounts of protein separated by SDS–PAGE and immunoblotted with anti-Numb (top), anti-LNX (middle) or anti-c-Cbl (bottom) to detect expression. (C) LNX-induced degradation of Numb is proteasome dependent. Twenty four hours after co-transfection of 293T cells with plasmids encoding Numb, LNX or empty vector, cells were incubated with dimethyl sulfoxide (DMSO) or the concentrations of MG132 indicated dissolved in DMSO for the times indicated. Whole-cell lysates were prepared and the equivalent amount of total protein was separated by SDS–PAGE. The levels of Numb expression were detected by immunoblotting with anti-Numb (top). Membranes were then stripped and blotted with anti-LNX to verify loading and LNX expression (bottom).

antiserum. Treatment of transfected cells with either 10 or 25 μM MG132 for 22 h effectively blocked the degradation of Numb (Figure 5C).

**LNX expression causes degradation of endogenous Numb**

To further assess the effect of LNX expression on endogenous Numb proteins, we transfected MDCK cells with wild-type or C48A LNX and HA-ubiquitin. Similar to the effect in 293T cells, Numb was ubiquitylated in the presence of wild-type but not mutant LNX (Figure 6A). Western analysis of total cell lysates with anti-Numb antibodies revealed that Numb protein levels were significantly reduced in the wild-type but not C48A transfected cells (Figure 6B). To visualize Numb protein levels in individual transfected cells, we stained MDCK cells transfected with either wild-type (Figure 6C, D and E) or C48A (Figure 6F, G and H) LNX with anti-Numb antiserum. LNX transfected cells were identified by anti-FLAG staining (Figure 6D and G) and Numb protein was detected by co-staining with anti-Numb (Figure 6C and F) and analysed by immunofluorescence and confocal microscopy. Merged images are shown in Figure 6E and H. In both non-transfected cells and cells transfected with mutant LNX (Figure 6C and F, shown in red), endogenous
Numb staining was observed in vesicular structures, which we and others have identified as early endosomes (Santolini et al., 2000; S.E.Dho, C.A.Smith and C.J.McGlade, in preparation). In cells transfected with wild-type LNX (green), Numb staining was selectively lost (Figure 6C and merged image in E). In order to assess whether LNX overexpression had a non-specific effect, we also stained LNX transfected cells with anti-SH C antisera...
Fig. 7. LNX expression enhances Notch nuclear activity. (A) CHO cells were transfected with ΔEC-Notch, a Hes-1 luciferase reporter construct and either empty pEF or pEF-LNX. Included in each transfection was a CMV β-galactosidase construct as a control for transfection efficiency. Total DNA was made up to 1.5 µg with empty parental vectors. Luciferase and β-galactosidase activities were measured from cell extracts 48 h after transfection. The percentage activation was calculated relative to ΔEC-Notch with empty vector as 100% activation. The data represent the mean ± SD of three independent experiments each performed in triplicate. (B) CHO cells were transiently co-transfected with pMT HA-ubiquitin and either pFLAG-CMV-2 or FLAG-tagged LNX<sup>WT</sup> or LNX<sup>ΔN</sup>. Numb protein was immunoprecipitated from ~1 mg of transfected cell lysate with anti-Numb antisemur and immunoblotted with anti-HA monoclonal antibody. (C) CHO cells were transfected with FLAG-tagged LNX<sup>WT</sup>. Cells were then co-stained with anti-FLAG and goat anti-mouse antibody conjugated to AlexaFlour488 to detect LNX transfected cells (green) and anti-Numb was detected by goat anti-rabbit antibody conjugated to CY3 (red). The merged image is shown at the bottom. Images were obtained by confocal microscopy.

(Figure 6I–K) to determine the effect of LNX over-expression on other endogenous proteins. SHC staining was unaltered in the wild-type LNX transfected cells, confirming the specificity of the effect of LNX on Numb protein levels.

Numb functions as an antagonist of the Notch signalling pathway and levels of Numb protein have been shown to modulate Notch-mediated activation of downstream targets such as Hes-1 (Frise et al., 1996; Guo et al., 1996; Spana and Doe, 1996; Artavanis-Tsakonas et al., 1999; Wakamatsu et al., 1999). Therefore we tested whether LNX expression and the consequent decrease in Numb protein levels could alter Notch-mediated activation of a Hes-1 luciferase reporter. A constitutively active form of Notch1 (ΔEC-Notch) activated Hes-1 luciferase when transfected into CHO cells (Figure 7A). Co-transfection of ΔEC-Notch with LNX resulted in ~30% increase in Notch nuclear activity, as measured by Hes-1 promoter-driven luciferase activity (Figure 7A). LNX expression in CHO cells also caused ubiquitylation of endogenous Numb (Figure 7B) and significant diminution of Numb staining (Figure 7C), suggesting that LNX reduces levels of Numb proteins resulting in augmentation of Notch signalling. We cannot formally exclude the possibility that LNX acts directly on Notch, although this seems unlikely since LNX expression does not promote Notch ubiquitylation or alter Notch protein expression levels (data not shown).

Discussion
Targeted protein degradation controls the levels of regulatory proteins involved in multiple cellular and developmental processes (Hershko and Ciechanover, 1998). The specificity of protein ubiquitylation is determined by the E3 components, which function as adaptors that selectively bind substrates (Tyers and Wilems, 1999; Joazeiro and Weissman, 2000). Several distinct classes of E3s have been defined, based on both their structural characteristics and modes of action.
Fig. 8. LNX belongs to a family of RING and PDZ domain-containing proteins. Schematic representation of p80 LNX and the highly related human protein (DDBJ/EMBL/GenBank accession No. CAC37490), the LNX p70 isoform and a human orthologue (AF257782) and related proteins semcap 3A and 3B and a related human protein (KIAA1095).

We have shown that LNX is a member of a growing family of RING finger-containing proteins that function as E3 ubiquitin ligases. Searches of the sequence databases identified a highly related sequence (DDBJ/EMBL/GenBank accession No. CAC37490) that is predicted to encode a protein, LNX2, which also contains a RING finger and four PDZ domains (Figure 8). Human orthologues of both LNX and LNX2 are also predicted within the human genome and are found at 4q12 and chromosome 13, respectively. In addition, two mouse (accession Nos AAF22131 and AAF22132) and one human sequence (accession No. KIAA1095) found in DDBJ/EMBL/GenBank encode related proteins containing a RING finger and two PDZ domains (Figure 8). With the exception of LNX, none of these proteins has been characterized yet. However, their common structural elements suggest that they probably belong to a family of related ubiquitin ligases.

Substrate recognition by E3s is achieved, in some cases, by the juxtaposition of specific protein–protein interaction modules and RING finger or HECT domains within the same polypeptide. For example, HECT domain-containing E3s of the Nedd4 family contain multiple WW domains, which bind to specific PY motifs contained within the substrate targets (Jackson et al., 2000; Rotin et al., 2000). The RING finger domain containing ubiquitin ligase c-Cbl recognizes tyrosine-phosphorylated substrates via its divergent SH2 domain (Joazeiro et al., 1999). LNX is the first example of an E3 ligase that appears to involve PDZ domains for substrate recognition.

PDZ domains are conserved protein modules that were originally identified as a 90–100 amino acid repeat in several cell junction proteins including PSD-95 (a synaptic protein), Drosophila discs large [a membrane-associated guanylate kinase (MAGUK) found at septate junctions] and ZO-1, another MAGUK protein associated with tight junctions in epithelial cells (Kennedy, 1995). PDZ domains are commonly found in multiple copies, in proteins that function as scaffolds for multiprotein signalling complexes or in complexes important for the establishment and maintenance of cellular organization and polarity (Sheng and Sala, 2001). PDZ domain-containing proteins such as PAR-3/Bazooka have also been identified as essential for asymmetric cell division in Caenorhabditis elegans and Drosophila (Bilder, 2001).

Our data suggest that both the Numb PTB domain-binding motif and the first PDZ domain of LNX are required for efficient substrate recognition and ubiquitylation. While the other three PDZ domains appear to be dispensable for binding and ubiquitylation of Numb, it seems likely that these domains could mediate the interaction with additional substrates of LNX. Proteins with PDZ domains often interact with the cytoplasmic tails of transmembrane proteins and localize to discrete submembranous sites. Therefore, the LNX PDZ domains may be involved in the recognition of transmembrane receptors, which may in turn be substrates for ubiquitylation by LNX. Interestingly, the related SEMCAP3A/B proteins were identified as molecules that interact with the cytoplasmic tail of transmembrane proteins called semaphorins. Recognition of specific transmembrane proteins by the LNX PDZ domains may also play a role in the subcellular localization of LNX. The identification of LNX PDZ domain binding partners should clarify the role of these domains in LNX function.

In Drosophila, Numb functions as an intrinsic determinant of cell fate by antagonizing the Notch signalling pathway (Uemura et al., 1989; Ryu et al., 1994). Levels of Notch signalling influence gene transcription and ultimately cell fate decisions (Artavanis-Tsakonas et al., 1999). One way in which levels of Numb protein and therefore Notch signalling are regulated is through the process of asymmetric cell division (Guo et al., 1996). During asymmetric cell division, Numb protein is localized to one pole of the dividing cell and the cleavage plane positioned such that Numb is segregated to one daughter cell (Knoblich et al., 1995). A daughter cell that receives Numb adopts a different fate to its sister. Such asymmetric localization of Numb has also been reported in the ventricular zone of the avian and mammalian central nervous systems (Zhong et al., 1996; Wakamatsu et al., 1999).

While some cell fate determinants like Numb and Notch are conserved from worm to mammals, a number of distinct strategies are used by different organisms to achieve asymmetric localization of these factors. In Drosophila, asymmetric localization of Numb prior to cell division is a consequence of recruitment and anchoring of Numb protein at a specific cortical site by proteins such as PON and Miranda (Lu et al., 1998, 1999; Shen et al., 1998). In mammalian systems, the mechanism that gives rise to asymmetric distribution of Numb is unknown and so far orthologues of Pon and Miranda have not been described. Our results demonstrate that mNumb protein levels are regulated by ubiquitin-dependent proteolysis and therefore one mechanism to generate asymmetric localization of Numb is through asymmetric or localized degradation. The presence of multiple PDZ domains in LNX would be consistent with a model in which asymmetric or polarized distribution of LNX could establish asymmetric distribution of Numb protein.

Materials and methods

cDNA constructs and methodologies

All wild-type and mutant LNX constructs were generated by PCR amplification of a mouse LNX cDNA template and ligated into the expression vectors indicated. The C48A point mutation in LNX was generated by PCR-directed mutagenesis. The LNX truncation mutants used in this study were LNXPAPDZ (amino acids 8–210), LNXA234 (amino acids 3–374), LNXA34 (amino acids 3–485) and LNAX4 (amino acids 3–628), and were also generated using PCR. All cDNAs generated
by PCR were verified by DNA sequencing. The p72 Numb isoform cDNA was subcloned into pcDNA3.1.

**Production of GST fusion proteins**

pGEX constructs were expressed in the BL21 strain of *Escherichia coli*. Expression of the GST fusion protein was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 3–5 h. The bacterial pellets were sonicated in phosphate-buffered saline (PBS) plus protease inhibitors and 0.1% Triton X-100 was then added. The cleared bacterial lysate was incubated for 1 h at 4°C with glutathione-Sepharose (GS) beads (Amersham Pharmacia Biotech), which were then washed four times with PBS.

The GS-bound material was resuspended in 500 μl of PBS, 1 mM dithiothreitol (DTT) and stored at −80°C. The concentrations of the GS-bound fusion proteins were determined by taking an aliquot of the protein for SDS–PAGE, staining with Coomassie Blue and comparing against bovine serum albumin (BSA) standards.

**In vitro ubiquitination assay**

Ubiquitination assays, unless indicated, were carried out using 0.6–1.0 μg of GS-bound GST fusion proteins, 2 μl of E2-containing lysate, 500 mM E1 from yeast (Affinity UW 8545, 2 mM ATP, 5 μM ubiquitin (Sigma–Aldrich U6253), 1 mM creatine phosphate (Sigma–Aldrich 27920), 7.5–15 μl E1, 1 mM creatine phosphate (Sigma–Aldrich 27920), 50 mM Tris–HCl pH 7.4, 2.5 mM MgCl₂ and 0.5 mM DTT. The mixture was incubated at room temperature for 90 min and the reaction was stopped by adding 2× SDS sample buffer. The reaction mixture was resolved by SDS–PAGE and analysed by western blot analysis using mouse anti-ubiquitin monoclonal antibody (Chemicon MAB1510). [35S]methionine-labelled Numb was synthesized by in vitro translation in wheat germ lysates using the TNT kit (Promega). Aliquots (5 μl) of in vitro translated Numb proteins were added into each in vitro ubiquitination reaction.

The E2 lysate used was prepared from E.coli BL21 transformed with histidine-tagged UbcH5B DNA in pT7-7 following induction with 1 mM IPTG. The bacteria were lysed by sonication in PBS plus protease inhibitors and the clarified supernatant used as the source of E2 was aliquoted and stored at −20°C.

**Cell culture and transfection**

MDCK, CHO and human 293T cell lines were grown to 50–70% confluence in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum on 10 cm dishes. DNA (1–2 μg) was added to the cells with LipofectAMINE in Opti-MEM. Following incubation at 37°C for 4 h, the DNA–LipofectAMINE solution was replaced with DMEM containing 10% serum. Whole-cell lysates were prepared 24 h post-transfection for immunoprecipitation and immunoblot analysis.

**Immunoprecipitations and western blotting**

Cell lysates were prepared from cultured cells grown to 70–90% confluence on 10 cm tissue culture dishes. Following two washes with cold PBS, the cells were scraped into 1 ml of HNTG-ZE lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1% Triton X-100, 100 mM glycerol, 100 mM ZnCl₂, 2 mM EDTA and protease inhibitors) and centrifuged at 14 000 g to pellet the insoluble matter. For each immunoprecipitation, 200–800 μl of lysate were made up to 1 ml with HNTG-ZE lysis buffer and incubated with antibody at 4°C for 2–4 h. Anti-Numb and anti-LNX polyclonal antisera were generated and affinity purified as previously described (Verdi et al., 1996; Dho et al., 1998, 1999). The immune complex was bound to protein A–Sepharose beads, washed three times with HNTG-ZE lysis buffer by boiling in SDS sample buffer. Proteins were separated by SDS–PAGE, transferred onto polyvinylidene fluordifluoride (PVDF) membranes (Immobilon-P) and immunoblotted with the primary antibodies indicated for 1–2 h at room temperature. Bound antibodies were visualized using goat anti-mouse in conjunction with the ECL system (Amersham Pharmacia Biotech).

For immunoprecipitation of ubiquitylated endogenous Numb, 293T cells were lysed in 1 ml of modified HNTG-ZE lysis buffer which contains 1% SDS and then boiled at 95°C for 5 min. In an experiment that used a proteasome inhibitor, transfected cells were incubated overnight with 20 μM MG132 before harvest. The lysates were then diluted with 10 ml of HNTG-ZE lysis buffer and subjected to immunoprecipitation with anti-HA antibody absorbed in protein G–Sepharose. After washing the absorbed beads with HNTG-ZE buffer, the immunoprecipitates were separated by SDS–PAGE and then immunoblotted with anti-Numb antibody.

**Immunocytochemistry and confocal microscopy**

MDCK or CHO cells were seeded onto sterile glass cover slips in 24-well cell culture dishes. Following attachment, the cells were transfected by LipofectAMINE 2000. Cells were fixed, 24 h post-transfection, in freshly prepared 4% paraformaldehyde in PBS at room temperature for 30 min and then permeabilized with 0.2% Triton X-100 in PBS for 10 min. Fixed cells were then incubated in blocking solution (5% normal goat serum and 2% BSA in PBS) for 1 h, followed by staining with appropriate primary antibodies (1:1000 dilution of anti-Flag M5 mouse monoclonal antibody; 1:500 dilution of polyclonal rabbit anti-LNX antisem; 1:200 dilution of polyclonal rabbit anti-Numb antisem; 1:500 dilution of polyclonal anti-SHC antisem) in blocking solution for 1 h at room temperature. Subsequently, cells on coverslips were washed three times with PBS and incubated with either Alexa 488-conjugated goat anti-rabbit, Alexa 488-conjugated goat anti-mouse or Cy3-conjugated donkey anti-rabbit for 1 h in the dark at room temperature. Coverslips were washed three times, mounted onto glass slides and analysed by confocal microscopy.

**Notch nuclear transactivation assay**

CHO cells seeded on 6-well plates were transfected with a membrane-tethered constitutively active form of mammalian Notch1, AEC-Notch (400 ng) and Hes-1 luciferase reporter construct (200 ng) in the presence and absence of pEF-LNX (800 ng). Included in each transfection was a cytomegalovirus (CMV) β-galactosidase construct (100 ng) as a control for transfection efficiency. Total DNA was made up to 1.5 μg with empty parental vectors. Cells were lysed 48 h after transfection in 1× reporter assay buffer (Promega), and luciferase and β-galactosidase activities were measured from cell extracts. Luciferase activity was quantitated using firefly luciferin according to the manufacturer’s instructions (Promega). β-galactosidase activity was determined using the chemiluminescent substrate, Galacto-Plus according to the manufacturer’s instructions (Tropix). Luciferase activity was normalized with β-galactosidase activity and represented as percentage activation of AEC-Notch alone. Each experiment was performed in triplicate and repeated at least three times.

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**References**


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