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c-Src is a PDZ interaction partner and substrate of the E3 ubiquitin ligase Ligand-of-Numb protein X1

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Abstract The very C-terminus of c-Src is a ligand for PDZ domains. In a screen for PDZ domains that interact with c-Src, we identified one of the PDZ domains of the Ligand-of-Numb protein X1 (LNX1), a multiple PDZ domain scaffold and RING type E3 ubiquitin ligase. We demonstrate that the interaction of c-Src with LNX1 depends on the C-terminal PDZ ligand of c-Src. Furthermore, we show that c-Src phosphorylates LNX1. Moreover, c-Src itself is ubiquitinated by LNX1, suggesting an interdependent regulation of c-Src and LNX1.

Keywords: LNX1; PDZ domain; Src protein kinase; Ubiquitination

1. Introduction

c-Src is a tightly regulated non-receptor tyrosine kinase. Overexpression or increased kinase activity of c-Src is frequently detected in human malignancies. c-Src is important for many cellular processes such as receptor-mediated signaling, cell adhesion, motility, cell proliferation and survival [1,2].

c-Src is composed of a C-terminal tail containing a negative-regulatory tyrosine residue (Tyr529), four Src homology (SH) domains and a unique amino-terminal domain (Fig. 1A) [1,2]. The SH1 tyrosine kinase domain contains the autophosphorylation site (Tyr418). The SH2 and SH3 protein–protein interaction domains interact with phosphotyrosine-containing motifs and proline-rich sequences, respectively. A short SH4 domain including a myristoyl group targets c-Src to membranes. Negative regulation of c-Src involves an intramolecular interaction between the SH2 domain and phosphorylated Tyr529, and of the SH3 domain with the SH1–SH2 linker region. This closed conformation is reversed by protein tyrosine phosphatases (PTP) and the interaction of the c-Src SH2 and SH3 domains with other proteins, which disrupt the inhibitory intramolecular interactions. Subsequently, c-Src becomes fully activated by autophosphorylation of Tyr418.

Recently, we described the very C-terminus of c-Src, GENL (Gly-Glu-Asn-Leu), as a ligand for the PDZ domain protein AF-6 [3]. PDZ domain proteins are multidomain proteins and function as scaffolds to organize cell adhesion complexes, to cluster transmembrane proteins, and serve as points of integration or divergence in signaling cascades [4]. In many cases PDZ proteins contribute to the maintenance of a non-proliferating state [5].

Ligand-of-Numb protein X1 (LNX1) was first identified as an interaction partner for the cell fate determinant and endocytic protein Numb [6]. LNX1 contains at its N-terminus a RING domain, which specifies LNX1 as an E3 ubiquitin ligase [7], followed by an amino acid motif NPAY for interaction with phosphotyrosine-binding (PTB) domains, and four PDZ domains, which mediate protein–protein interactions (Fig. 1A). LNX1 has been described as E3 ubiquitin ligase and endocytic scaffold, e.g. for Numb [8,9]. A shorter alternatively spliced isoform, p70LNX1, lacking the RING domain has been described in brain and kidney [6,10] and acts as an endocytic scaffold.

To further study the role of PDZ ligand-mediated interactions of c-Src, we performed a screen for PDZ domains that interact with c-Src. One candidate we identified was the PDZ3 domain of LNX1, a RING type E3 ubiquitin ligase. Here, we present biochemical and biological data on the interaction of c-Src with LNX1 and show their mutual regulation.

2. Materials and methods

2.1. Antibodies

Antibodies used: anti-FLAG (Sigma); anti-Src GD11, anti-phosphotyrosine (4G10) (Upstate); anti-c-Src (Src2), anti-myc (9E10), anti-tubulin (Santa Cruz); anti-Src (32G6) (Cell Signaling Technology). Polyclonal LNX1/55 and LNX1/56 affinity purified peptide antibodies were generated against aa 134–148 and aa 671–685, respectively (Eurogentec).

2.2. Plasmids

Human LNX1 was obtained as cDNA clone (RZPD, Germany; IRATp970C0541D; BC022983). The coding sequence was subcloned into pcDNA3 by PCR and fusing the FLAG-tag sequence 5′ to the coding sequence. FLAG-LNX1ΔPDZ, FLAG-LNX1-NT and FLAG-LNX1-CT constructs were generated by PCR mutagenesis. Src expression constructs were described previously [3]. The myc-Ubiquitin plasmid was a gift from Dr. Y. Yarden (Rehovot, Israel).

2.3. Cell culture and transfection

Cell culture and transient transfection were described elsewhere [3].

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Abbreviations: LNX1, Ligand-of-Numb protein X1; PDZ, PSD-95, postsynaptic density 95; Dlg, disc large; ZO-1, zonula occludens-1; SH, Src homology; PTB, phosphotyrosine binding; GST, glutathione-S-transferase

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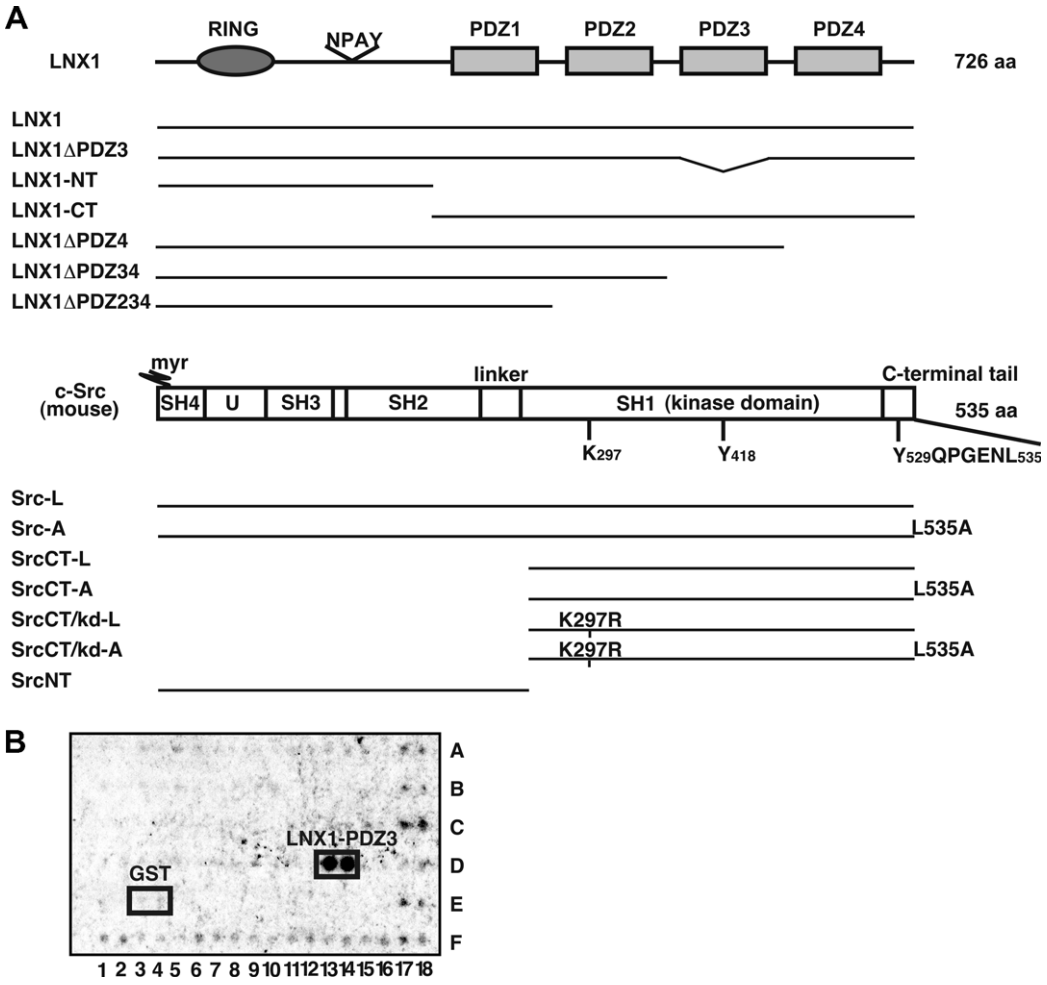

Fig. 1. c-Src interacts with LNX1 PDZ3. (A) Schematic representation of LNX1 and c-Src. Regions encoded by the different constructs are shown. (B) PDZ arrays were incubated with HEK 293 cell lysates overexpressing c-Src. Bound c-Src was detected by blotting with anti-Src.

2.4. PDZ domain arrays

TranSignal™ PDZ domain arrays were obtained from Panomics (Redwood City, USA) and incubated according to the manual with lysates of HEK 293 cells overexpressing c-Src or control protein. Bound c-Src was detected by blotting with anti-Src antibody.

2.5. Immunoprecipitations, immunoblots and immunofluorescence microscopy

Experiments were performed as described previously [3].

3. Results

3.1. c-Src interacts with the PDZ3 domain of LNX1

Recently, we described the C-terminal sequence GENL of c-Src as a PDZ ligand that binds to the PDZ domain of the cell junction protein AF-6 [3]. We assumed the existence of further PDZ domains that may interact with the C-terminal sequence of c-Src. To identify such PDZ domains, we screened PDZ domain arrays, which included a total of 123 different human PDZ domains. The membranes were incubated with lysates of human embryonic kidney (HEK) 293 cells overexpressing c-Src (Fig. 1B) or a control protein (data not shown). Bound protein was detected by blotting with a Src-specific antibody. We found that c-Src interacted with the PDZ3 domain of LNX1, the only of four PDZ domain of LNX1 present on the arrays.

3.2. PDZ-dependent interaction and co-localization of c-Src and LNX1

Next, we analyzed whether c-Src and full-length LNX1 can interact. HEK 293 cells were transiently transfected with plasmids encoding wild-type c-Src (Src-L) and FLAG-LNX1. Src-L coprecipitated with FLAG-LNX1, and FLAG-LNX1 coprecipitated with Src-L (Fig. 2A and B). A mutant of c-Src (Src-A) where the C-terminal hydrophobic amino acid Leu is replaced with Ala leading to impaired binding to PDZ domains [5] showed reduced coprecipitation with FLAG-LNX1 (Fig. 2A), and vice versa FLAG-LNX1 with Src-A (Fig. 2B). Thus, c-Src binds to LNX1 in a PDZ domain-dependent manner.

We next investigated the co-localization of c-Src and LNX1 upon coexpression in HeLa cells by confocal microscopy. Src-L was distributed throughout the cytoplasm, near the plasma membrane and at sites of cell–cell contacts (Fig. 2C, red). FLAG-LNX1 was diffusively distributed throughout the cell, including in the nucleus, and regions near the plasma membrane (Fig. 2C, green). We also observed localization of FLAG-LNX1 to microtubuli-like structures. Src-L and FLAG-LNX1 partially co-localized at membrane regions and cell-cell contacts (Fig. 2C, upper panel, arrow) and in membrane ruffles (Fig. 2C, lower panel). Hence, the co-localization of c-Src and LNX1 in specific subcellular compartments

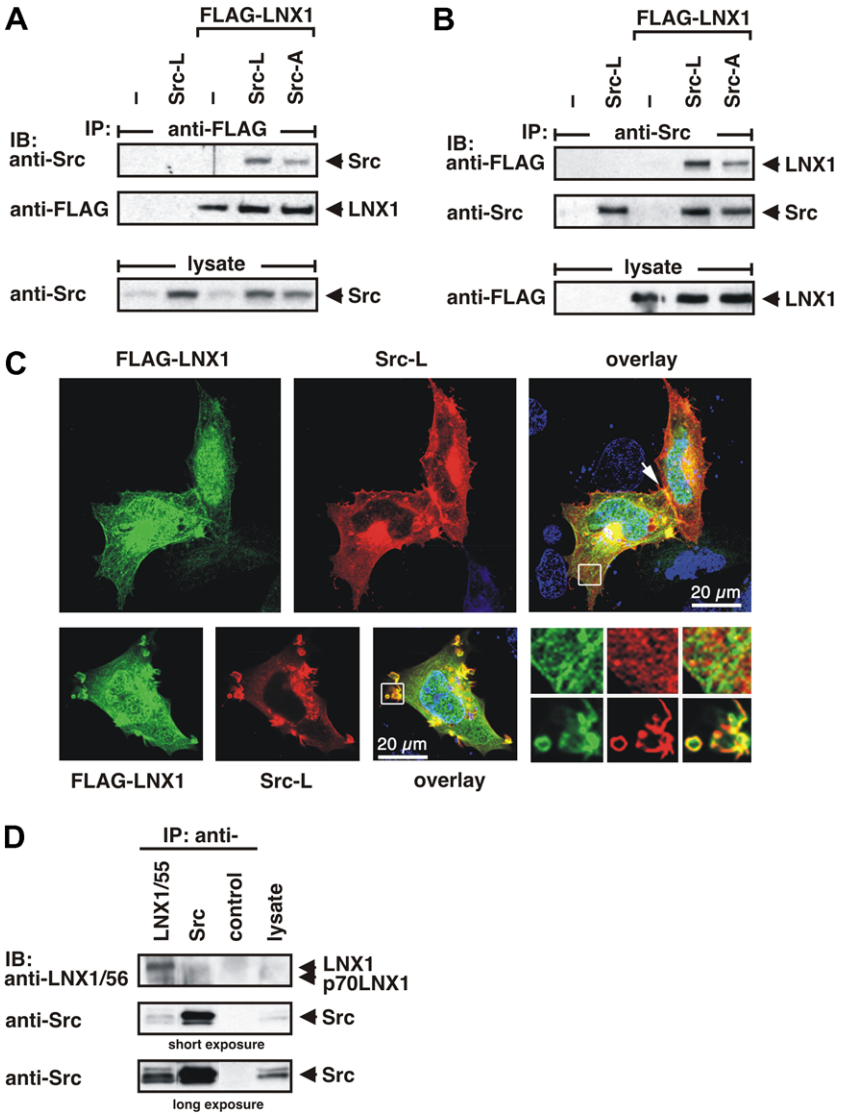

Fig. 2. Interaction of c-Src and LNX1. (A,B) Plasmids encoding FLAG-LNX1, Src-L, Src-A or empty vector (−) were transfected into HEK 293 cells as indicated. Cell lysates were immunoprecipitated (IP) with anti-FLAG (A) or anti-Src (B) and immunoblotted (IB) with anti-Src, or anti-FLAG. Equal expression of Src constructs was confirmed by blotting cell lysates with anti-Src. (C) Co-localization of c-Src and LNX1. HeLa cells were transfected with FLAG-LNX1 and Src-L, fixed and immunostained with rabbit anti-FLAG (green) and mouse anti-Src GD11 (red) antibodies. Single confocal sections in green and red channel and overlay thereof are shown. LNX1 and Src-L co-localize at cell–cell contacts (arrow) in punctae in the cytoplasm and in peripheral membrane ruffles (magnification of squared areas); bar is 20 μm. (D) Coprecipitation of endogenous c-Src and LNX1 from mouse brain extracts. Proteins were precipitated from extracts and blotted with the indicated antibodies.

provides additional evidence for the interaction between c-Src and LNX1.

To test whether c-Src and LNX1 also interact in vivo, we immunoprecipitated LNX1 from mouse brain lysate (Fig. 2D). LNX1 cannot be detected in whole brain lysate but is detected following immunoprecipitation. c-Src specifically coprecipitated with LNX1, indicating that the complex exists in vivo.

3.3. Mapping the interaction of c-Src and LNX1

We next examined the interaction sites for c-Src on LNX1. We coexpressed Src-L with FLAG-tagged full-length LNX1, LNX1ΔPDZ3, LNX1-NT or LNX1-CT (Fig. 1A). As before, Src-L coprecipitated with FLAG-LNX1, and to a similar extend with FLAG-LNX1-CT (Fig. 3). Src-L showed a reduced binding to FLAG-LNX1ΔPDZ3 and no binding to FLAG-LNX1-NT. In pulldown assays, we analyzed the interaction of GST-fusion proteins of individual PDZ domains with in vitro translated Src-L and SrcCT-L (C-terminus of c-Src harboring the PDZ ligand). For both proteins, we confirmed the interaction with GST-PDZ3 and observed a stronger interaction with GST-PDZ1, but no significant interaction with GST-PDZ2 and GST-PDZ4 (data not shown).

These data demonstrate that c-Src interacts with PDZ1 and PDZ3 domains of LNX1. Residual binding of c-Src to LNX1 indicates a contribution of PDZ domain-independent interaction.

3.4. Src-mediated phosphorylation of LNX1

The interaction between c-Src and LNX1 raised the question whether LNX1 was a substrate for Src kinase. Upon coexpression of FLAG-LNX1 and Src-L in HEK 293 cells, we observed

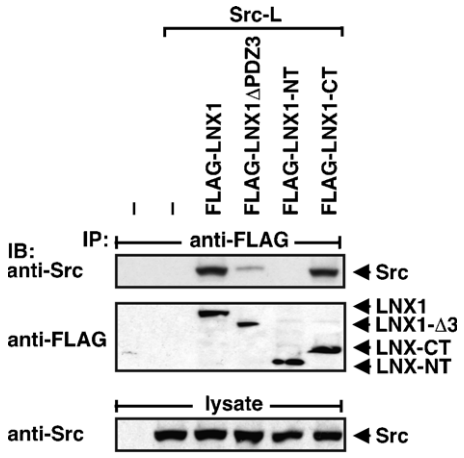

Fig. 3. Mapping the interaction of Src and LNX1. HEK 293 cells were transfected as indicated. LNX1 was precipitated with anti-FLAG and blotted with anti-Src. IB with anti-FLAG and anti-Src antibodies were used as control.

Src-dependent phosphorylation of LNX1, which was increased by treating cells with pervanadate, a potent PTP inhibitor (Fig. 4A). For further investigation, we expressed the constitutively activated SrcCT-L protein together with FLAG-LNX1-NT or FLAG-LNX1-CT. We detected Src-dependent phosphorylation of LNX1-NT and no significant phosphorylation of LNX1-CT (Fig. 4B).

3.5. LNX1-dependent ubiquitination of activated Src

We also asked whether the Src kinase activity plays a role for the c-Src/LNX1 interaction. We coexpressed SrcCT proteins with FLAG-LNX1 in HEK 293 cells. SrcCT-L bound very strongly to FLAG-LNX1, whereas the mutant SrcCT-A showed a markedly reduced interaction (Fig. 5A). The corresponding kinase-defective proteins SrcCT/kd-L and SrcCT/kd-A showed the same effect (Fig. 5A), indicating that the Src kinase activity does not affect the Src/LNX1 interaction. Interestingly, we also observed a Src kinase activity-dependent mobility shift of coprecipitated constitutively activated SrcCT-L, but not of kinase-defective SrcCT/kd-L (Fig. 5A). Therefore, we analyzed the interaction of SrcCT-L with FLAG-LNX1ΔPDZ deletion mutants (Fig. 1A) and the mobility shift of coprecipitated SrcCT-L. SrcCT-L binding to LNX1 and mobility shift of coprecipitated SrcCT-L increased with gradual C-terminal deletion of PDZ domains (Fig. 5B). In contrast, SrcCT-L only weakly bound to LNX1-NT and coprecipitated SrcCT-L was not shifted, indicating that PDZ1 is necessary for both, sufficient interaction and modification of SrcCT. Furthermore, shifted SrcCT proteins coprecipitated by LNX1 are phosphorylated at Tyr416 (data not shown).

This mobility shift depends on Src kinase activity (Fig 5A) and is characteristic of protein ubiquitination. We tested this by cotransfection of SrcCT-L and myc-Ubiquitin into HEK 293 cells with FLAG-LNX1 or FLAG-LNX1ΔPDZ234, the LNX1 mutant with increased ubiquitin ligase activity. We inhibited proteasomal degradation and protein synthesis by treating cells with MG132 and cycloheximide, and analyzed SrcCT-L proteins. Inhibitor treatment resulted in a smear of SrcCT cotransfected with LNX1, which was increased using LNX1ΔPDZ234 (Fig. 5C). This suggested LNX1-mediated

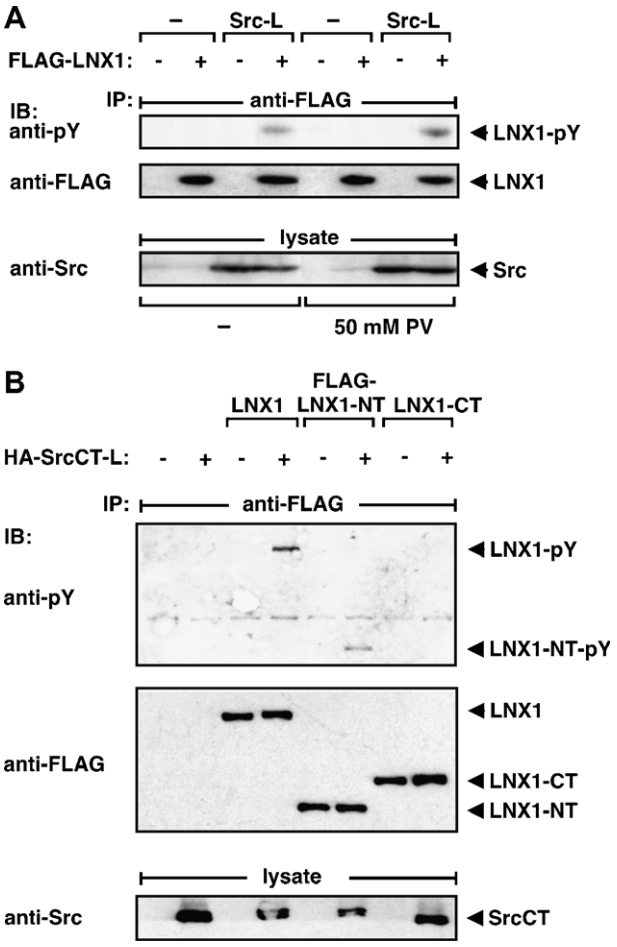

Fig. 4. Src-mediated phosphorylation of LNX1. HEK 293 cells were transfected as indicated. (A) Cells were treated with or without pervanadate (PV) for 15 min, and FLAG-LNX was precipitated with anti-FLAG and blotted with anti-phospho-tyrosine. Equal loading and expression was controlled using anti-FLAG and anti-Src antibodies. (B) Cells were lysed in presence of 0.5% SDS and 0.5% DOC and analyzed as under (A).

ubiquitination of SrcCT. To further prove this, we precipitated SrcCT-L and immunoblotted with anti-myc antibody to detect ubiquitinated SrcCT-L proteins. SrcCT-L was ubiquitinated and ubiquitination increased when LNX1 or LNX1ΔPDZ234 was coexpressed (Fig. 5D). Consistently, we observed decreased protein levels of constitutively activated full length SrcY527F when cotransfected with FLAG-LNX1 and myc-Ubiquitin (Fig. 5E). These data suggest that activated Src is ubiquitinated by LNX1 and targeted for degradation.

4. Discussion

The C-terminus of c-Src, GENL, is a ligand for PDZ domains and binds to the PDZ domain of AF-6 [3]. Most PDZ ligands show promiscuity and bind to different PDZ domains [4]. Using PDZ domain arrays, we searched for additional PDZ domains to interact with the c-Src ligand and identified the PDZ3 domain of LNX1. We also observed a binding of Src-L and SrcCT-L to GST-PDZ1 (data not shown). We demonstrated here that c-Src and LNX1 interacted within cells and

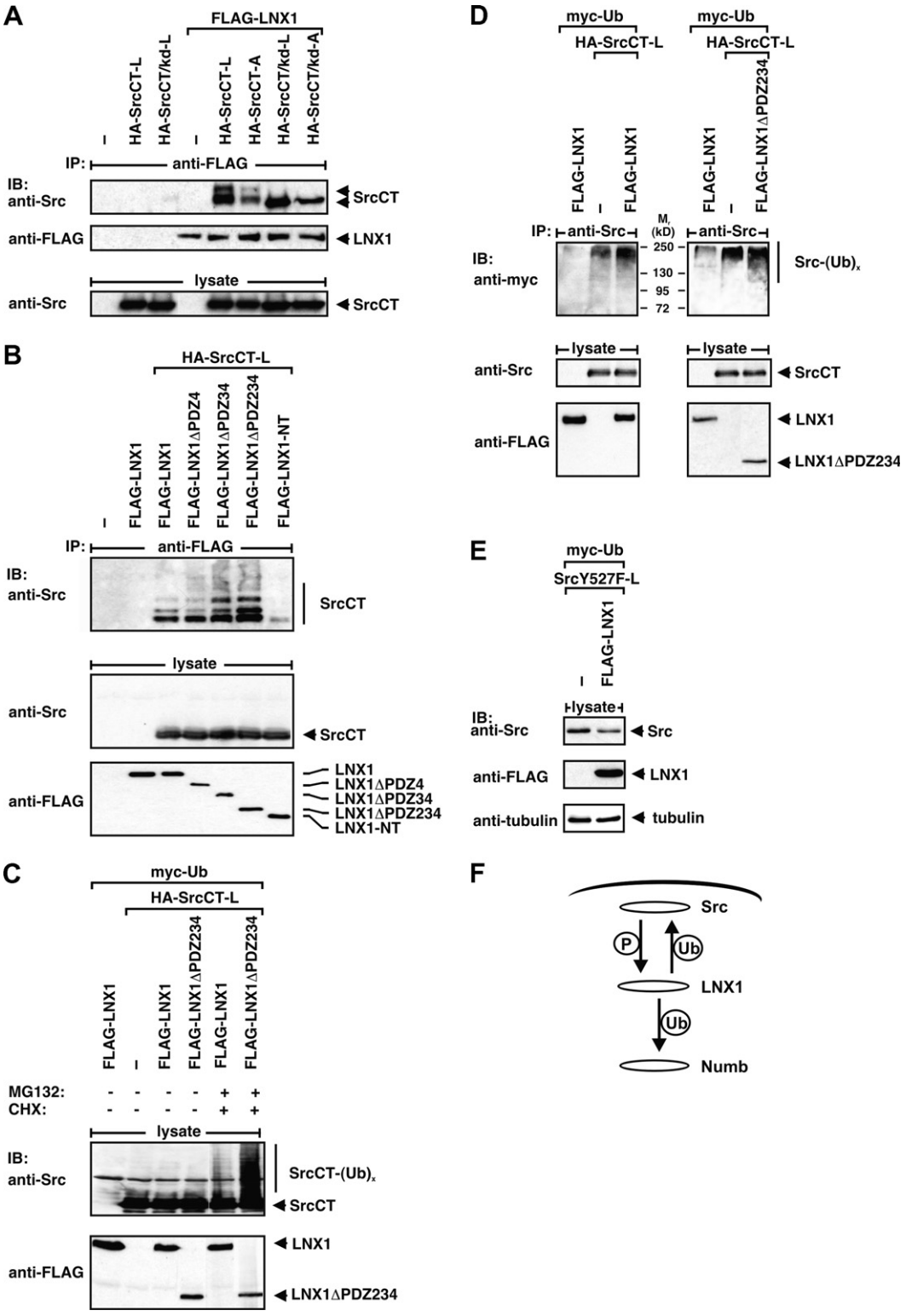

Fig. 5. LNX1-dependent ubiquitination of activated Src. (A–E) HEK 293 cells were transfected and lysates analyzed as indicated. (C) Cells were treated with or without 50 μM MG132 and 20 μg/ml cycloheximide (CHX) for 3 h prior to lysis. (F) Model of interaction between c-Src and LNX1. For details, see text.

in tissue (Fig. 2). Furthermore, using the Src-A mutant harboring a defective PDZ ligand, or the LNX1ΔPDZ3 mutant, we showed that this interaction was dependent on the c-Src PDZ ligand (Figs. 2A, B and 3).

Mutant Src-A showed weak binding to LNX1. This suggests that PDZ domain-independent interactions contribute to the c-Src/LNX1 interaction. Indeed, we observed a weak interaction of SrcNT with LNX1 (data not shown), and SrcCT-A

lacking the SH2, SH3 and SH4 domain of c-Src showed a reduced interaction with LNX1 compared to full length Src-A. However, we cannot exclude indirect interactions mediated by other proteins.

We also show here that c-Src co-localizes with LNX1 mainly in regions of cell–cell contacts and in peripheral membrane ruffles when coexpressed in HeLa cells (Fig. 2C). LNX1 acts as scaffold for Numb and interacts with transmembrane proteins such as ErbB2 [9,11].

We further showed that LNX1 is a target for c-Src-dependent phosphorylation and that phosphorylation sites lie mainly in the N-terminus (Fig. 4). However, we cannot exclude c-Src-induced phosphorylation by other tyrosine kinases. Phosphorylation of a PDZ domain protein can regulate the interaction with its PDZ domain binders [5,12]. Interestingly, coprecipitation of SrcCT-L protein with FLAG-LNX1 was independent of Src kinase activity. This indicates that Src-dependent LNX1 phosphorylation is not likely to play a role for the c-Src/LNX1 interaction, whereas it may regulate the function of LNX1.

Moreover, we observed Src kinase-dependent mobility shift of LNX1-bound SrcCT (Fig. 5A). Both, LNX1/SrcCT interaction and mobility shift of LNX1-bound SrcCT increased with gradual C-terminal deletion of PDZ domains (Fig. 5B). In contrast, LNX1-NT lacking all PDZ domains only weakly bound SrcCT and was not shifted, indicating the requirement for PDZ1. These findings are reminiscent of effects shown for Numb ubiquitination by the same LNX1 mutants [7]. Indeed, we could demonstrate LNX1-mediated ubiquitination of activated SrcCT by using MG132 and cycloheximide, which inhibit proteasomal degradation and protein synthesis, thereby enriching for ubiquitinated SrcCT proteins (Fig. 5C). These findings are strengthened by detection of increased SrcCT ubiquitination in the presence of LNX1 and LNX1ΔPDZ234 (Fig. 5D). We did not observe similar effects for c-Src. This may be due to weak kinase activity of c-Src under the experimental conditions used, whereas SrcCT is constitutively activated. However, the fact that the protein level of constitutively active SrcY527F is reduced by LNX1 coexpression (Fig. 5E) suggests that activated Src is degraded by LNX1-dependent ubiquitination.

Activated Src is a known substrate for the E3 ubiquitin ligase c-Cbl leading to degradation of Src [13]. In contrast to c-Cbl, binding of Src to LNX1 depends on its functional C-terminal PDZ ligand. We suggest that LNX1-associated Src is ubiquitinated by LNX1 in a kinase-dependent manner and that the interaction of c-Src and LNX1 could affect the capability of LNX1 to ubiquitinate other substrates such as Numb (Fig. 5F).

c-Src is regulated by different PDZ domain proteins in different way. Here, we show that the PDZ protein LNX1 negatively regulates activated Src by leading to ubiquitination and degradation. Previously, we demonstrated another mechanism for regulation of cellular c-Src kinase activity where binding of c-Src to the AF-6 PDZ domain holds c-Src in a partially active form and restricts substrate phosphorylation [3], a mechanism we did not observe for binding to LNX1. These two mechanisms may differ by being irreversible or reversible. The combined loss of these regulatory mechanisms in oncogenic v-Src and c-Src mutants found in some human tumors, both lacking the C-terminal sequence, is likely to contribute to the constitutive active nature of these kinases.

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