# Identification of LNX as a novel Src-interacting protein

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## Abstract

Overexpression or increased activity of the proto-oncogene *c-src* is frequently detected in human malignancies. The non-receptor tyrosine kinase c-Src is important for many cellular processes such as receptor-mediated signaling, cell adhesion, motility, cell proliferation and survival. However, the precise functions of c-Src in normal tissues and in cancer remain unclear.
Recently, we described the very C-terminus of c-Src, GENL (Gly-Glu-Asn-Leu), as a class III ligand for the PDZ domain protein AF-6 (see abstract Radziwill et *al.*). 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 of signaling cascades. In many cases PDZ proteins contribute to the maintenance of a non-proliferating state.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 to interact with c-Src is a PDZ domain of the Ligand-of-Numb protein X (LNX). LNX is a scaffold and a RING type E3 ubiquitin ligase, which contains, in addition to its RING domain, an NPXY motif and four PDZ domains. Here, we report the interaction of c-Src with LNX. By using a mutant where the C-terminal Leu of c-Src is mutated to Ala, a mutation that was shown to abolish binding of the ligand to a PDZ domain, we demonstrate that this interaction is at least in part PDZ-dependent. Furthermore, we show that LNX is a substrate for Src-mediated phosphorylation. Expression of LNX leads to the ubiquitination of its target proteins. Co-expression of c-Src and LNX, however, interferes with LNX-mediated ubiquitination.

## Schematic representation of LNX1 and c-Src

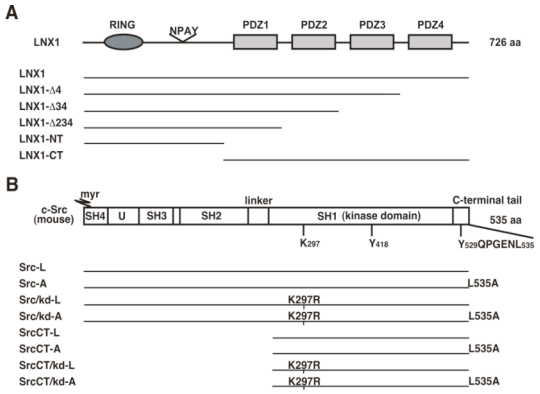


Fig. 1: Schematic representation of LNX1 (A) and c-Src (B). Ligand-of-Numb protein X1 (LNX1) consists of a RING (really interesting gene) domain, a NPAY motif and 4 PDZ (PSD-95 / Dlg-1 / ZO-1) domains. Cellular (c-) Src is composed of an amino-terminal Src homology (SH) 4 domain attached with a myristoyl group (myr), a unique region (U), SH3 and SH2 protein-protein interaction domains, a tyrosine kinase domain (SH1) that contains Lys297 and Tyr418 (Tyr416 in chicken), and a carboxy-terminal regulatory domain that contains Tyr529 (Tyr527 in chicken) and a PDZ binding sequence (GENL). The regions encoded by different LNX1 and c-Src constructs are shown.

## LNX1 interacts with c-Src

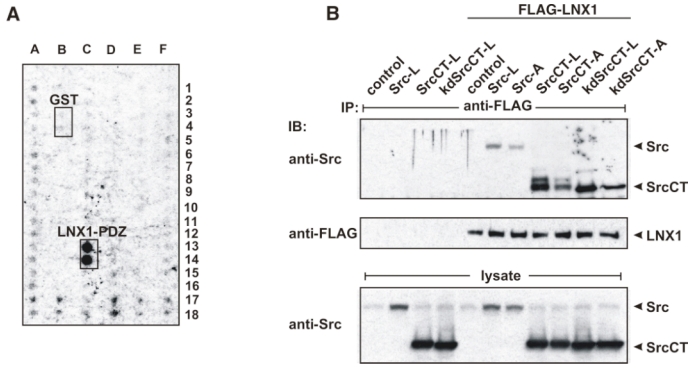


Fig. 2: (A) PDZ arrays were incubated with HEK 293 cell extracts overexpresing c-Src. Bound c-Src was detected by immunoblotting the membranes with anti-Src. (B) Plasmids encoding FLAG- LNX1, Src-L, Src-A, SrcCT-L, SrcCT-A, kinase dead (kd) SrcCT-L or kdSrcCT-A were transfected into HEK 293 cells as indicated. Cells were lysed, and cell extracts were immunoprecipitated (IP) with mouse anti-FLAG and immunobotted (IB) with anti-Src to show the partially PDZ- dependent interaction of LNX1 and c-Src, or with anti-FLAG to control equal loading. Equal expression of Src constructs was demonstrated by immunoblotting cell extracts with anti-Src.

## c-Src mediates tyrosine phosphorylation of LNX1

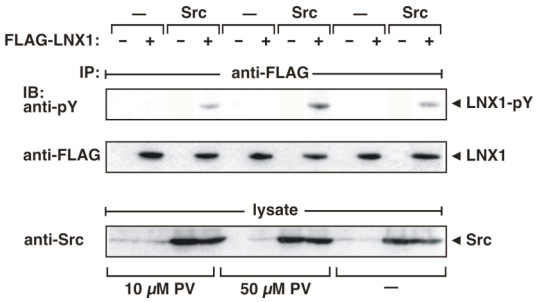


Fig. 3: Plasmids encoding c-Src, FLAG-LNX1, or both were transfected into HEK 293 cells. After 24 hr, the cells were stimulated with pervanadate (PV) for 15 min, and cell extracts were immunoprecipitated with mouse anti-FLAG and immunobloted with anti-phospho-tyrosine to show the tyrosine phosphorylation of LNX1.

## Influence of c-Src on LNX1 ubiquitin ligase activity

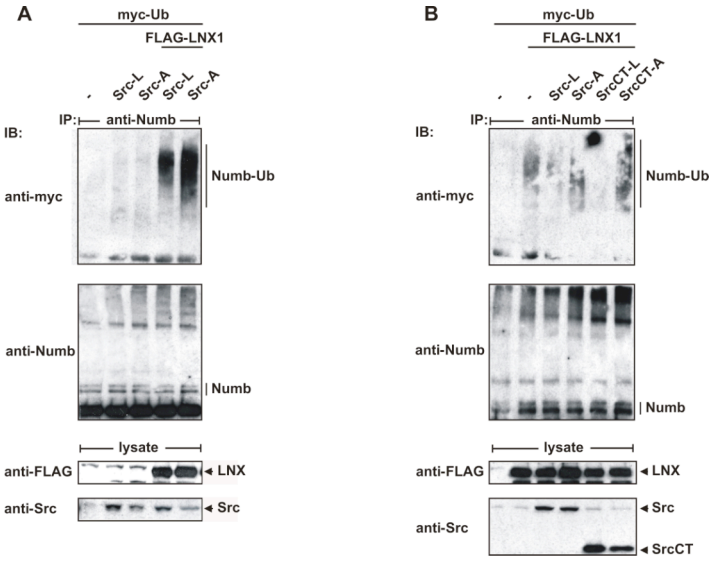


Fig. 4: Plasmids encoding myc-Ub, FLAG-LNX1, Src-L, Src-A, SrcCT-L or SrcCT-A were transfected into HEK 293 cells as indicated. After 36 hr, the cells were lysed, immunoprecipitated with rabbit anti-Numb and immunobotted with anti-myc to show Numb ubiquitylation, and anti-Numb to control equal loading. Cell extracts were immunoblotted with anti-FLAG and anti-Src to control equal expression.

## Model for c-Src as a regulator of the LNX ubiquitin ligase activity

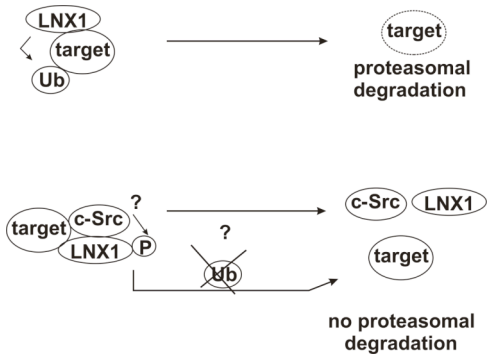


Fig. 5: LNX1 can act as an E3 ubiquitin ligase. Binding of LNX1 to its target proteins leads to their ubiquitination and subsequent proteasomal degadation. Upon interaction with c-Src, LNX1 becomes phosphorylated. Furthermore, the ubiquitination of target proteins by LNX1 is reduced. The negative regulatory effect of c-Src on LNX1-mediated ubiquitination can directly be dependent on LNX1 phosphorylation or on sequestration or inhibition of the LNX1 / target complex by c-Src.

## Summary

Recently, we described the very C-terminus of c-Src as a class III ligand for the PDZ domain protein AF-6 (see abstract Radziwill et *al.*). In a screen for PDZ domains that interact with c-Src we identified a PDZ domain of LNX to bind to c-Src. Upon co-expression, we could show that c-Src co-precipitates with LNX whereas a c-Src mutant that was shown to be abolished in its ability to bind to a PDZ domain co-precipitates in a lesser extend. This suggests that the c-Src / LNX interaction is at least in part dependent on the C-terminal PDZ ligand of c-Src and indicates a general function of the very C-terminus of c-Src as a PDZ domain binding sequence.
LNX1 is composed of several domains including a RING domain and four PDZ domains, suggesting a role of LNX1 as E3 ubiquitin ligase and molecular scaffold. We demonstrated that LNX1 is a substrate for Src-dependent phosphorylation. Furthermore we could show, that overexpression of c-Src interferes with the ability of LNX1 to sufficiently ubiquitinate its endogenous target proteins. The mechanism of the inhibitory effect of c-Src on the LNX1-mediated ubiquitination and the contribution of the PDZ-dependend c-Src / LNX1 interaction is under investigation.