LNX/PDZRN E3 Ubiquitin Ligase Family

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The ligand of Numb protein-X (LNX) family, also known as the PDZRN family, is composed of four discrete RING-type E3 ubiquitin ligases (LNX1, LNX2, LNX3, and LNX4), and LNX5 which may not act as an E3 ubiquitin ligase owing to the lack of the RING domain.

LNX   PDZRN   RING   PDZ domain,E3 ubiquitin ligase,UPS,lysosomal pathway,disease

1. Introduction

Protein ubiquitylation, which is a highly conserved post-translational modification in which ubiquitin (consisting of 76 amino acids) is covalently linked to lysine residues of a substrate protein, is associated with nearly all aspects of eukaryotic physiology. The number of genes encoding proteins involved in ubiquitin-dependent post-translational modification in humans is estimated to be over 1000, which far exceed the total number of kinase-encoding genes (518) identified thus far[1][2]. Protein ubiquitylation comprises a series of enzymatic reactions that initially uses energy from ATP hydrolysis and is mediated by ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3), which ultimately render substrate specificity[3][4]. E3 ubiquitin ligases can be classified into three groups based on the presence of discrete domains. While HECT family of E3 ubiquitin ligases acts as a genuine enzyme by exploiting its catalytic cysteine residue located in C-terminal HECT domain, the E3 ligase with RING or U-box domain serves as a scaffold that recruits the E2 enzyme in close proximity to the bound substrate. While protein turnover mediated by the UPS is essential for cellular proteostasis, nonproteolytic ubiquitin modification is important for the regulation of epigenetic control, transcriptional regulation, cellular trafficking, and post-translational modification[5]. The presence of extracellular 20S proteasome in blood plasma and cancer cell culture conditioned media indicates the complexity of proteostasis[6]. Therefore, it is not surprising that UPS dysfunction is associated with multiple human diseases. Thus far enormous efforts have been dedicated to understanding the pathophysiological roles of individual E3s because the complexity of the UPS mostly stems from multiple substrates or diverse cellular roles of a single substrate of an E3 ubiquitin ligase[7][8].

Here we describe one of the multifunctional E3 ubiquitin ligases family, ligand of numb protein-X (LNX/PDZRN). Among the five members of the LNX family (LNX1-LNX5), LNX1-LNX4 may function as E3 ubiquitin ligases that target miscellaneous substrates for ubiquitylation, as these possess a RING domain with multiple PDZ (PSD95, DLGA, ZO-1) domains, each of which are considered protein-protein interacting modules[9][10][11][12][13]. Although over 600 E3 ubiquitin ligases are encoded in the human genome[1], LNX proteins belong to the sole E3 ligase family with multiple PDZ domains (up to four); therefore, it is not surprising that members of the LNX family appear to interact promiscuously with various different proteins[14][15][16][17]. Among the LNX proteins, two were

originally named LNX1 (also called PDZRN2) and LNX2 (PDZRN1) owing to their ability to bind to NUMB, an intracellular Notch inhibitor[18] via relatively well-conserved NUMB-binding motifs (NPAY in LNX1, NPAF in LNX2) located between the RING domain and the first PDZ domain; the consensus sequences from these are not present in other members of the LNX family. Thus LNX3, LNX4, and LNX5 are more prone to be referred to as PDZRN3, PDZRN4, and PDZRN5 (or PDZRN4L), respectively though (PDZRN; PDZ and RING)[9][17][19][20][21], we will collectively call the several synonyms of LNX/PDZRN/SEMCAP as LNX hereafter.

Although considerable efforts have been devoted during the last two decades to elucidating the function of LNXs, our knowledge of their substrates, binding partners, pathophysiological functions, and detailed molecular mechanisms of action of LNX proteins remains elusive. As an indication of the increased interest in this area of research, an excellent review article published in 2018[17] comprehensively discussed the role of LNX1 and LNX2 in various diseases. Therefore, in this review, after primarily emphasizing novel discoveries on LNX1 and LNX2 in the last three years, we will focus on findings of the cellular and pathogenic roles of LNX3 and LNX4, and then discuss what major questions remain to be tackled.

2. Structural Significance and Sequence Similarity of Members of the LNX/PDZRN E3 Ubiquitin Ligase Family

Although the complete X-ray crystallographic structure of LNX proteins is not available, fragmented structures of LNX1p80 and LNX2 are published on various web sites and in the literature. According to previous reports based on the available X-ray crystallographic structures, the N-terminal three-dimensional structures of LNX1 and LNX2 show the presence of a single RING domain with two Zn finger motifs flanking the ends of the RING domain (Zn-RING-Zn fragment), and LNX2 s PDZ domain[22][23][24]. Structural information on the other parts of individual PDZ domains of LNX1 (PDZ2 and PDZ3) are also available in the Protein Data Bank (PDB; http://www.rcsb.org). In 2018 Young[17] provided a detailed account of the structural information of LNX1 and LNX2 that is available. The predicted amino acid sequences and primary structures of human LNX proteins, with domain annotations, are shown in Figure 1. RING-type E3 ubiquitin ligases tend to form homodimers or heterodimers to acquire enzymatic properties, though some act as monomers[25]. Although both LNX1 and LNX2 Zn-RING-Zn fragments could be homodimerized, homodimerization is exclusively required for LNX1 to function as an E3 ubiquitin ligase, while the monomeric status of LNX2 is sufficient for the retention of its auto-ubiquitylation activity[22][24].
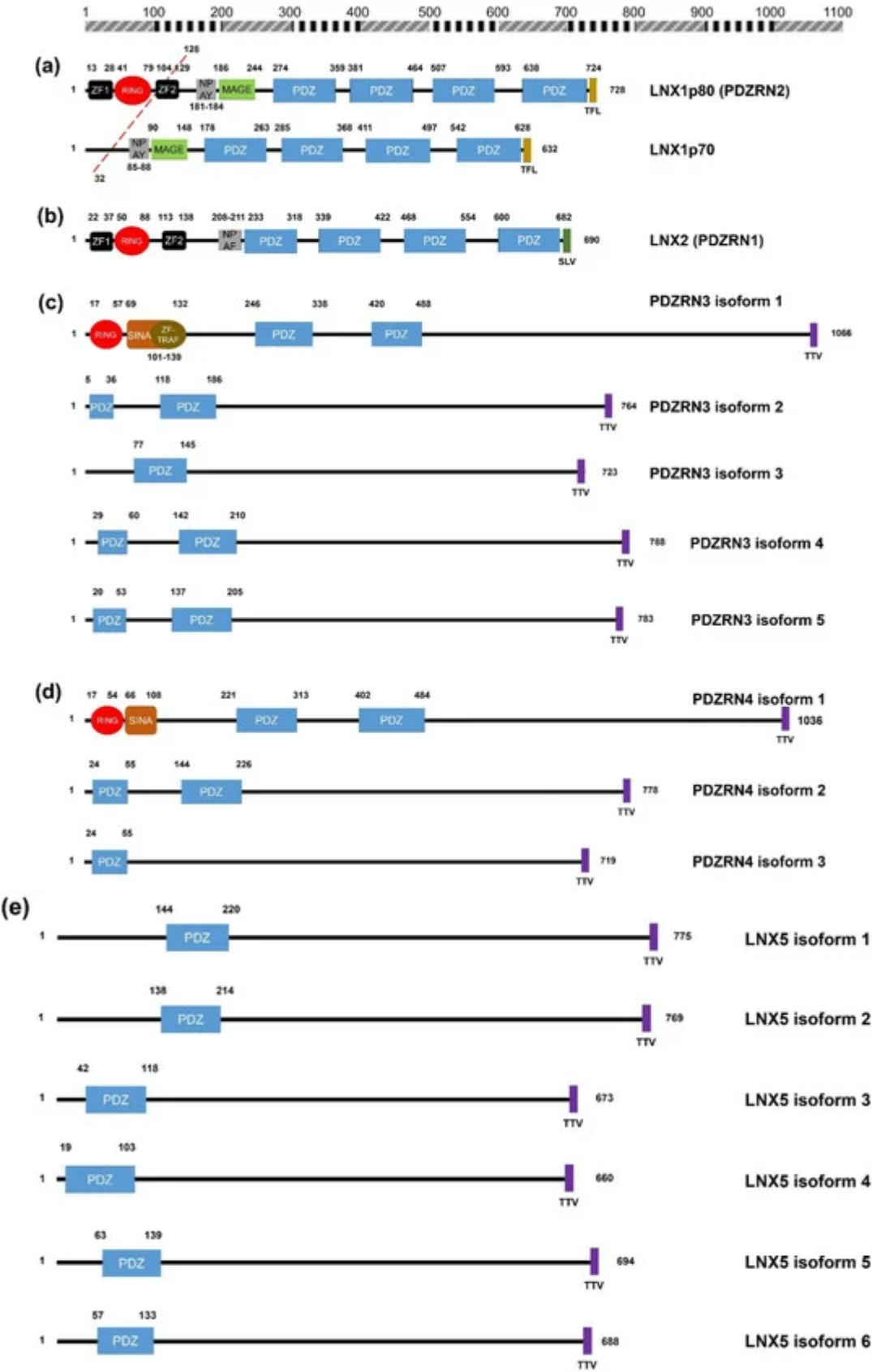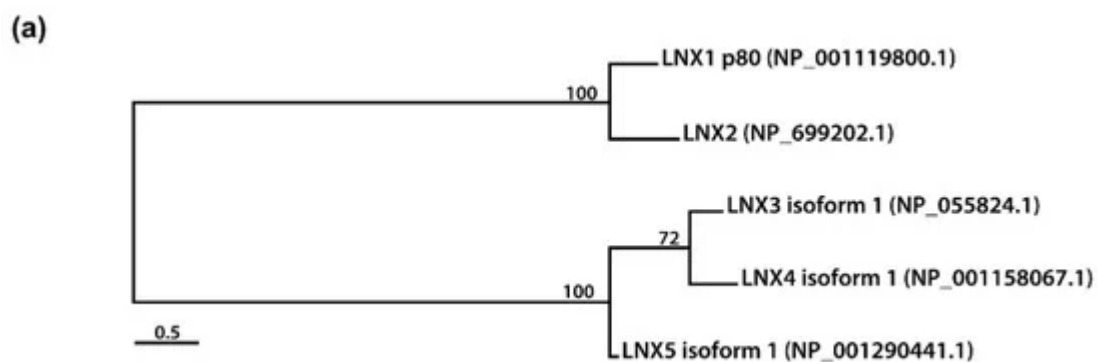
Figure 1. Schematic diagrams of the members of the human LNX family. (**a**) LNX1 has two splicing variants, LNX1p70 and LNX1p80. In contrast to the longer splicing variant, the shorter one does not contain the N-terminal RING domain and the two neighboring Zn finger motifs, while the other known domains following the second Zn

finger motif are conserved, which include four PDZ domains and a NUMB-binding motif. Both variants bind to NUMB via the NPAY motif and contain a MAGE-B interacting region. The red dashed bar indicates the point from which the amino acid sequences become identical. (**b**) LNX2 showed the highest similarity with LNX1p80 in terms of primary structure. However, the major difference between LNX1p80 and LNX2 was noted at the region located between the NUMB-binding motif and the point of initiation of the first PDZ domain. While LNX1p80 has a relatively longer sequence that includes the MAGE-B18-interacting motif, LNX2 only has a 21-amino acid-spanning region. (**c**) LNX3 has five splicing variants. The PDZRN4 isoform 1 is the sole variant equipped with an N-terminal RING domain and a juxtaposed Zn finger flanking the C-terminal end of the RING domain. All the variants have two PDZ domains, except the third isoform, which only has one PDZ domain. (**d**) Among LNX4 variants, which include three isoforms, only the PDZRN4 isoform 1 has an N-terminal RING domain and a SINA-like motif. Similar to LNX3, the variants have two PDZ domains; the third isoform is an exception as it has only one relatively short PDZ domain identical to that in the PDZRN4 isoform 3. (**e**) LNX5 has six splicing variants. All variants have a single PDZ without any recognizable domains. The last three single letter amino acid codes represent the class I PDZ-binding motif (S/T-X-V/I/L). The scale bar on the top indicates the approximate length in terms of the number of amino acids for the comparison of individual proteins. ZF; Zn finger motif, RING; really interesting new gene, MAGE; melanoma-associated antigen, PDZ; PSD95-DLGA-ZO-1, SINA; seven in absentia, TRAF; tumor necrosis factor receptor (TNF-R)-associated factor. The schematic illustration of the alignment of the LNX/PDZRN domains are derived from the deduced amino acid sequence information retrieved from GenBank; the corresponding accession numbers have been provided: LNX1p80 (NP_001119800.1), LNX1p70 (NP_116011.2), LNX2 (NP_699202.1), PDZRN3 isoform 1 (NP_055824.1), PDZRN3 isoform 2 (NP_001290068.1), PDZRN3 isoform 3 (NP_001290069.1), PDZRN3 isoform 4 (NP_001290070.1), PDZRN3 isoform 5 (NP_001290071.1), PDZRN4 isoform 1 (NP_001158067.1), PDZRN4 isoform 2 (NP_037509.3), PDZRN4 isoform 3 (EAW57827.1), LNX5 isoform 1 (NP_001290441.1), LNX5 isoform 2 (NP_115901.2), LNX5 isoform 3 (NP_001290442.1), LNX5 isoform 4 (NP_001290443.1), LNX5 isoform 5 (NP_001290444.1), LNX5 isoform 6 (NP_001290445.1).

The possible formation of an oligomer or heteromer between LNX1 and LNX2 may be considered if we take into account the existence of multiple PDZ domains and class I PDZ-binding motifs (S/T-X-V/I/L) at the C-termini of both proteins (Figure 1a,b). The oligo- or heterodimeric complex formation was validated in yeast two-hybrid experiments, where the presence of C-terminal class I PDZ-binding motifs were a prerequisite for studying their intermolecular interaction[9]. However, whether the Zn-RING-Zn domains of both LNX1 and LNX2 can mediate heterodimerization has not been investigated thus far. Collectively, the dimerization sites in LNX1 and LNX2 may exist in at least two different positions: in the N-terminus containing the RING and Zn finger motifs, and in the PDZ domains. The proposed concepts were strongly supported by the finding that while the introduction of multiple mutations in the gene encoding the isolated Zn-RING-Zn domain was adequate to disrupt homodimerization of the fragments, when introduced in the genes encoding full-length LNX1 and LNX2 the same mutations failed to prevent dimerization [22][24]. Additionally, the intramolecular loop of the C-terminal class I PDZ-binding motifs could also have bound to the second PDZ domains (class I PDZ domain is able to bind S/T-X-C) of LNX1 and LNX2[15]. Therefore, their intrinsic E3 ubiquitin ligase functions were regulated at the intramolecular level, and could be further modulated by post-translational modifications, such as by the attachment of phosphate moieties. Thus far,

only a single protein kinase (c-Src) has been reported to mediate LNX1p80 phosphorylation[26]. In addition, since LNX family besides LNX5 has several Zn ion coordination sites, it would be encouraged to establish the optimal level of Zn ion to achieve as fully active recombinant proteins as possible in vitro by following well established experimental procedures[27][28].

Besides LNX5, the remaining members of the LNX family (PDZRN3 and PDZRN4) contain an N-terminal RING domain and two tandem repeat PDZ domains (Figure 1c–e). While PDZRN3 contains a Zn finger motif at the C-terminal end of its RING domain[29], the presence of this motif in PDZRN4 could not be confirmed, although PDZRN4 does contain a SINA-like domain that may incorporate a Zn ion. Interestingly, the sequences of all members of the LNX family (except LNX1 and LNX2), including their variants, end with a TTV segment, which is also a type of class I PDZ-binding motif. Therefore, LNX1 and LNX2 might interact with other LNX proteins via their PDZ domains and C-terminal class I PDZ-binding motifs. Alternative assumptions might be also possible, considering the structural homology could be inferred from the level of amino acid sequence similarity. Strikingly by pairwise comparison of the individual members of LNX/PDZRN family (Figure 2), we only observed the high sequence similarities between LNX1 and LNX2, and LNX3 and LNX4. While LNX1 and LNX2 as their names imply share some substrates (Table 2) including Numb for ubiquitin dependent modification, LNX3 (PDZRN3) and LNX4 (PDZRN4) though their overlapping targets has not been reported yet would possibly be complementary to each other at least in part under the particular cellular contexts by sharing common substrates. To clarify the unique or overlapping molecular and cellular functions of the individual LNX proteins, their mutually complementary roles on possible target proteins must be scrutinized through future studies.
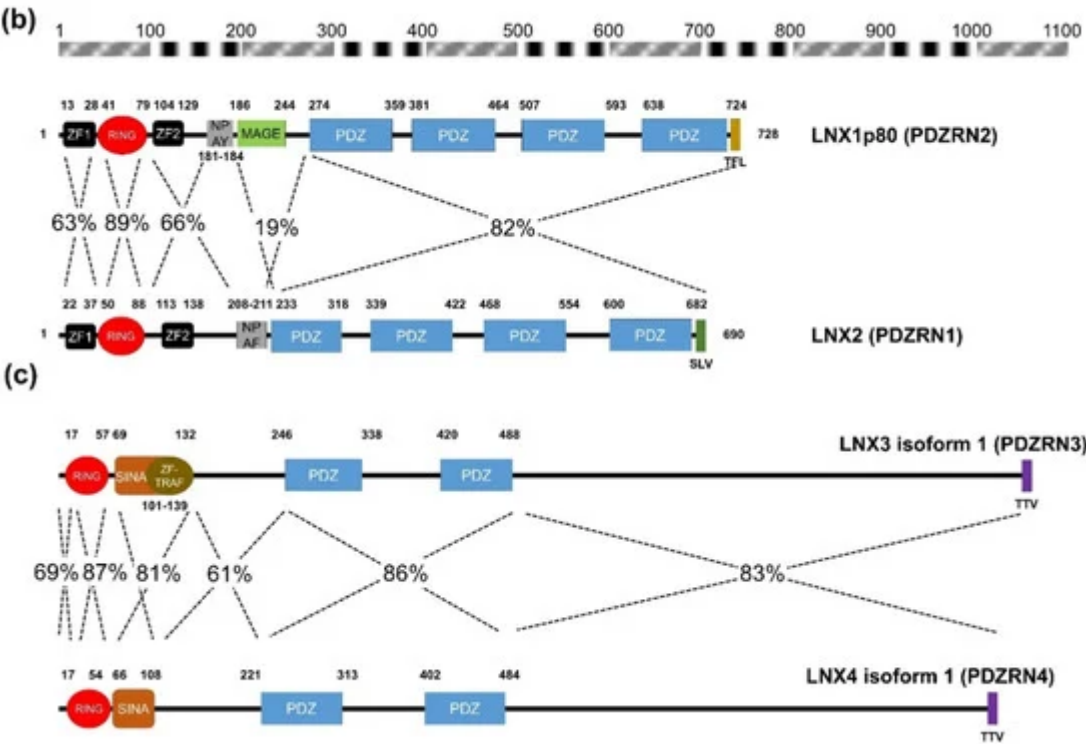
Figure 2. Amino acid sequence comparison of human LNXs (**a**) Phylogenetic tree of the LNX proteins. Maximum likelihood analysis was performed using to RAxML under the LG+GAMMA model. Analysis was carried out with five LNXs encoding the longest of their own variants (LNX1p80, LNX2, LNX3 isoform 1, LNX4 isoform 1, and LNX5 isoform 1). The numbers on each node represent bootstrap supported values. The scale bar indicates the number of substitutions/site. (**b**,**c**) Homology comparison of each domains was highlighted by the value of percentage. (**b**) Pairwise comparison between LNX1 and LNX2. The scale bar on the top indicates the approximate numbers of amino acids for the comparison of individual proteins. (**c**) Pairwise comparison between LNX3 (PDZRN3) and LNX4 (PDZRN4).

Table 2. Newly discovered LNX1 and LNX2 associated proteins in the past three years (2018–2020).

Associated Proteins	LNX	Domain Involved	Methods Used	Major Functions	Consequences	References
NEK6	LNX1	RING and 3rd PDZ	Co-IP, GST pull down, Yeast two hybrid,	Serine/threonine kinase	K48-linked polyubiquitylated NEK6 undergoes UPS-dependent degradation	[31] (2020)
LDOC 1	LNX1		Co-IP	Tumor suppressor	LNX1 uses LDOC1 as a scaffold protein to indirectly target phospho-JAK2 destruction	[32] (2019)
MDM2	LNX1		Co-IP	E3 ubiquitin	LNX1 may indirectly	[37](2019)

Associated Proteins	LNX	Domain Involved	Methods Used	Major Functions	Consequences	References
				ligase	interact with MDM2	
p53	LNX1		Co-IP	Tumor suppressor	LNX1 indirectly mediates p53 destruction	[37](2019)
Connexin 36	LNX1	2nd PDZ	Co-IP, GST pull down, Ni-NTA pull down	Gap junction protein	Ubiquitylated connexin36 undergoes lysosomal-dependent degradation.	[50] (2018)
Connexin 36	LNX2	2nd PDZ	Co-IP, Ni-NTA pull down	Gap junction protein	Ubiquitylated connexin36 undergoes lysosomal-dependent degradation.	[50](2018)
EphB1	LNX1 p70	N-terminal region ahead of 1st PDZ domain	Co-IP	Receptor tyrosine kinase	Stabilization	[56](2018)
EphB2	LNX1 p80			Receptor tyrosine kinase	Degradation	[56](2018)
EphB2	LNX1 p70	2nd PDZ	Co-IP, GST-pull down	Receptor tyrosine kinase	Stabilization	[56](2018), [57] (2019)
GluN1	LNX1		Co-IP	Glutamate receptor subunit	LNX1 helps form a NMDAR complex by recruiting GluN1 and GluN2B	[57](2019)
GluN2B	LNX1	1st PDZ	Co-IP, GST pull down	Glutamate receptor subunit	LNX1 helps form a NMDAR complex by recruiting GluN1 and GluN2B	[57](2019)
GlyT2	LNX1, LNX2	2nd PDZ	Co-IP	Glycine transporter	Polyubiquitylated and degraded	[58](2019)

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