

Ligand of Numb proteins LNX1p80 and LNX2 interact with the human glycoprotein CD8 α and promote its ubiquitylation and endocytosis

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Accepted 13 June 2011

Journal of Cell Science 124, 3545–3556

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doi: 10.1242/jcs.081224

Summary

E3 ubiquitin ligases give specificity to the ubiquitylation process by selectively binding substrates. Recently, their function has emerged as a crucial modulator of T-cell tolerance and immunity. However, substrates, partners and mechanism of action for most E3 ligases remain largely unknown. In this study, we identified the human T-cell co-receptor CD8 α -chain as binding partner of the ligand of Numb proteins X1 (LNX1p80 isoform) and X2 (LNX2). Both *LNX* mRNAs were found expressed in T cells purified from human blood, and both proteins interacted with CD8 α in human HPB-ALL T cells. By using an in vitro assay and a heterologous expression system we showed that the interaction is mediated by the PDZ (PSD95-DlgA-ZO-1) domains of LNX proteins and the cytosolic C-terminal valine motif of CD8 α . Moreover, CD8 α redistributed LNX1 or LNX2 from the cytosol to the plasma membrane, whereas, remarkably, LNX1 or LNX2 promoted CD8 α ubiquitylation, downregulation from the plasma membrane, transport to the lysosomes, and degradation. Our findings highlight the function of LNX proteins as E3 ligases and suggest a mechanism of regulation for CD8 α localization at the plasma membrane by ubiquitylation and endocytosis.

Key words: CD8, E3 ligase, LNX protein, Endocytosis, Ubiquitylation

Introduction

The localization of transmembrane receptors at the plasma membrane (PM) is dynamically controlled by both the rate of delivery via the exocytic pathway and the rate of internalization and transport to the endosomes via endocytosis. Intrinsic protein-sorting signals and cellular machineries able to decode them regulate traffic along the two routes. Alteration in these mechanisms affects proper receptor function at the PM and is often correlated to pathologies (reviewed in Benado et al., 2009; Mellman and Nelson, 2008; Stenmark, 2009).

One of the sorting signals for proteins along the exo- and endocytic pathways is the ubiquitylation, a post-translational modification by which ubiquitin, a polypeptide of 76 amino acid residues, is covalently attached to lysine (Lys) residues of a substrate protein (Hershko and Ciechanover, 1992). Once thought to only mediate proteasomal degradation in the cytosol (Pickart and Fushman, 2004), ubiquitylation might also occur at the PM where it regulates protein internalization, at the trans-Golgi complex where it directs proteins to the late endosomes, and in endosomes to sort proteins to the multivesicular bodies (reviewed in Mukhopadhyay and Riezman, 2007; Piper and Luzio, 2007). In all these cases, it results in protein degradation into the lysosomes. The fate and the transport of the tagged substrate will depend on the Lys residue involved in the formation of ubiquitylation chains, as well as on the number of residues added. Single ubiquitin monomers can be attached to one or several Lys

of a protein (mono- or multimonoubiquitylation, respectively). Ubiquitin itself possesses several Lys residues that can be used for the attachment of another ubiquitin molecule, allowing substrates to be modified with different types of ubiquitin chains (polyubiquitylation). Ubiquitylation occurs in a stepwise manner involving three enzymes: an ubiquitin-activating enzyme E1 is responsible for the attachment of free ubiquitin; a second ubiquitin-conjugating enzyme E2 receives it from E1; and a third ubiquitin ligase E3 catalyzes the final transfer of the ubiquitin from the E2 enzyme to the substrate. The specificity of the ubiquitylation process is determined by the E3 ligases, which are suggested to function as an adaptor to bind substrates selectively. More than 600 E3 ligases were found in the human proteome and were classified into two major groups, defined by the presence of either a HECT (homologous to the E6 associated protein C terminus) or a RING (really interesting new gene) domain as catalytic domain.

Recently, it has been emerging that E3 ligases play an important function in regulating T-cell tolerance and immunity (reviewed in Bhoj and Chen, 2009; Deshaies and Joazeiro, 2009; Gomez-Martin et al., 2008). For instance, the E3 ligase c-Cbl regulates T-cell receptor (TCR) activation by both degrading key signalling molecules such as PLC γ -1 (Jeon et al., 2004) and controlling TCR internalization and transport to the lysosomes (Naramura et al., 2002). Interestingly, the defective expression of

some E3 ligases has been related to the development of autoimmune diseases, such as encephalomyelitis, arthritis and autoimmune diabetes (Chiang et al., 2000; Gronski et al., 2004; Jeon et al., 2004). However, to date, our knowledge of the substrates, partners, biological functions and mechanism of action for most E3 ligases remains elusive.

The T-cell co-receptor CD8 is a type I transmembrane protein expressed as $\alpha\alpha$ homodimer on the surface of intestinal T cells, $\gamma\delta$ T cells, thymic T-cell precursors and NK cells, and as $\alpha\beta$ heterodimer on thymocytes and peripheral T cells (Gangadharan and Cheroutre, 2004; Irie et al., 1995). The surface expression of CD8 $\alpha\beta$ heterodimer depends on the α -chain (Goldrath et al., 1997). Efficient surface expression of the α -chain requires its cytosolic C-terminal valine motif (C-TVM), a ligand of PDZ domains that modulate its delivery to the PM by sequentially interacting with GRASP65 and GRASP55 proteins (D'Angelo et al., 2009; Iodice et al., 2001). Required for the activation of cytotoxic T lymphocytes, CD8 stabilizes the interaction between the TCR on the surface of the lymphocytes and the class I major histocompatibility complex on antigen-presenting cells. Furthermore, it recruits the p56^{lck} protein tyrosine kinase, bound to the cytosolic tail of its α -chain, to the vicinity of the TCR. As such, CD8 functions as coactivator, because Lck is a major proximal effector of the T cell activation cascade (Salmond et al., 2009; Weiss and Littman, 1994). Development of cytotoxic T lymphocytes was

greatly reduced in mice lacking CD8 α (Fung-Leung et al., 1991), and defects in CD8 α and CD8 β expression were shown to correlate with pathological conditions such as immunodeficiencies and Wiskott-Aldrich syndrome (de la Calle-Martin et al., 2001; Kawabata et al., 1996; Schmitz et al., 1998). How CD8 function is regulated at the PM and whether and how its impairment leads to immune diseases is still largely unknown.

In this study, we were looking for proteins that could modulate CD8 α localization at the PM by using CD8 α cytosolic tail as bait in a two-hybrid assay. We identified and characterized the interactions between CD8 α and LNX1p80 and LNX2, which result in CD8 α ubiquitylation, internalization in the endocytic pathway and degradation. We also demonstrated that LNX proteins are expressed in the same physiological context as CD8 α and interact with it in human HPB-ALL T cells. Thus, our data strongly suggest that the E3 ubiquitin ligase activity of both LNX proteins is involved in the regulation of CD8 α localization at the PM.

Results

CD8 α binds the LNX proteins LNX2 and LNX1p80

In order to identify novel interacting-proteins involved in the regulation of CD8 α intracellular trafficking, we performed a yeast two-hybrid screening using a human liver cDNA library and the CD8 α cytosolic C-terminal tail as bait (Fig. 1A). Among

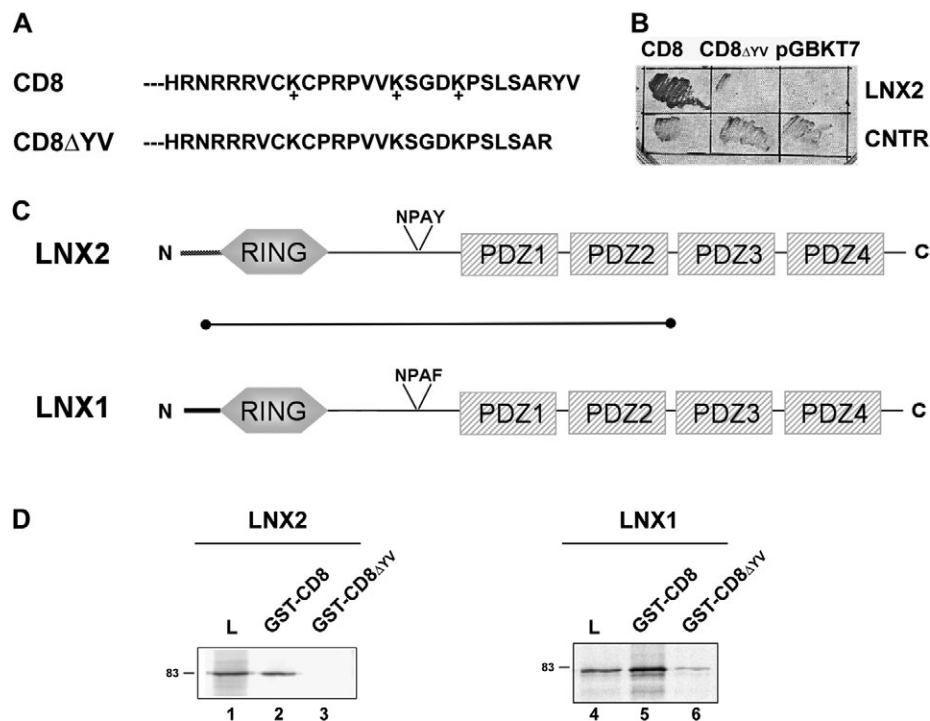


Fig. 1. Identification and characterization of LNX2 and LNX1p80 as interacting partners of CD8 α . (A) Amino acid sequences of CD8 α wild-type and Δ YV mutant cytosolic C-terminal tail used as baits in the two-hybrid assay. The + symbol indicates lysine residues where ubiquitin chains might be attached.

(B) Identification of the N-terminal fragment of LNX2 by two-hybrid assay: the yeast colony expressing the N-terminal fragment was able to grow on a high-stringency selection plate when co-transformed with plasmid expressing the CD8 α wild-type cytosolic tail. No growth was detected when either a control plasmid (pGBKT7, vector with the GAL4 DNA binding domain) or a plasmid expressing the Δ YV-mutated tail was co-transformed. CNTR represents yeast colony transfected with the vector expressing only the GAL4 activation domain, which grew at low extent in all the transformations. (C) Representation of LNX2 and LNX1p80. LNX proteins contain a RING finger domain, an NPAY or NPAF motif for interaction with Numb protein and four PDZ domains. The line underneath LNX2 indicates the fragment obtained by two-hybrid assay. (D) In vitro interaction between either LNX2 or LNX1p80 and the cytosolic tail of CD8 α .

Glutathione-Sepharose beads, loaded with GST fused to CD8 α wild-type (lanes 2 and 5) or Δ YV mutant cytosolic tail (lanes 3 and 6), were incubated with LNX2 (lanes 2 and 3) or LNX1p80 (lanes 5 and 6) that had been labelled with [³⁵S]methionine and translated in vitro. Bound proteins were eluted from the beads and analyzed together with the loaded samples (L, lanes 1 and 4) by SDS-PAGE and autoradiography. Numbers on the left indicate the molecular mass (kDa).

positive clones, one contained a cDNA fragment encoding amino acids 56–451 of the LNX2 protein (Fig. 1B). LNX2 was initially identified as a ligand of the endocytic protein Numb (ligand of Numb protein X) (Dho et al., 1998; Rice et al., 2001). It was classified as a RING-type E3 ligase because it contains an N-terminal RING finger domain (Fig. 1C). However, it has been poorly characterized and its function as E3 ligase has never been addressed. In addition, it also contains four PDZ domains, presumably mediating protein–protein interactions. The cDNA fragment identified by the two-hybrid assay corresponded to the LNX2 N-terminal region including the RING finger domain, the NPAY motif for the interaction with Numb, and the first two PDZ domains (Fig. 1C). Interestingly, the interaction was disrupted when the PDZ ligand signal was removed from the bait by deletion of the terminal Tyr and Val residues from the C-TVM (CD8- Δ YV) (Fig. 1B), indicating that the binding between CD8 α and LNX2 is strongly dependent on this signal.

To confirm the interaction between LNX2 and CD8 α , LNX2 cDNA was transcribed in vitro and translated. The radioactively labelled protein was incubated with either the CD8 α cytosolic C-terminal tail or its Δ YV-mutant fused to GST (see Materials and Methods). The bound proteins were separated by SDS-PAGE

and revealed by autoradiography. As shown in Fig. 1D, LNX2 associates in vitro with the cytosolic C-terminal tail of CD8 α (Fig. 1D, lane 2), but not with its Δ YV mutant (Fig. 1D, lane 3).

LNX2 is closely related to the p80 isoform of LNX1, another member of the LNX protein family (Fig. 1C) (Dho et al., 1998; Rice et al., 2001). However, in contrast to LNX2, the function of LNX1p80 as E3 ligase has been partially explored (Kansaku et al., 2006; Takahashi et al., 2009). For these reasons, we decided to test whether LNX1 is also a CD8 α binding protein. On the other hand, we were not surprised that this protein was not found in our two-hybrid assay because LNX1p80 is poorly expressed in human liver cells (Dho et al., 1998). As observed for LNX2, in vitro transcribed and translated LNX1p80 was also able to interact with the CD8 α cytosolic C-terminal tail fused to GST (Fig. 1D, lane 5). The removal of the C-TVM from CD8 α decreased the interaction (Fig. 1D, lane 6).

CD8 α protein is usually present in T and NK cells, but whether LNX1p80 or LNX2 is expressed in the same cell type has never been described. Therefore, we purified a T- and NK-cell-enriched fraction from human blood (see Materials and Methods) and performed RT-PCR by using primers specific for mRNAs encoding LNX1p80 and LNX2. Both genes were efficiently

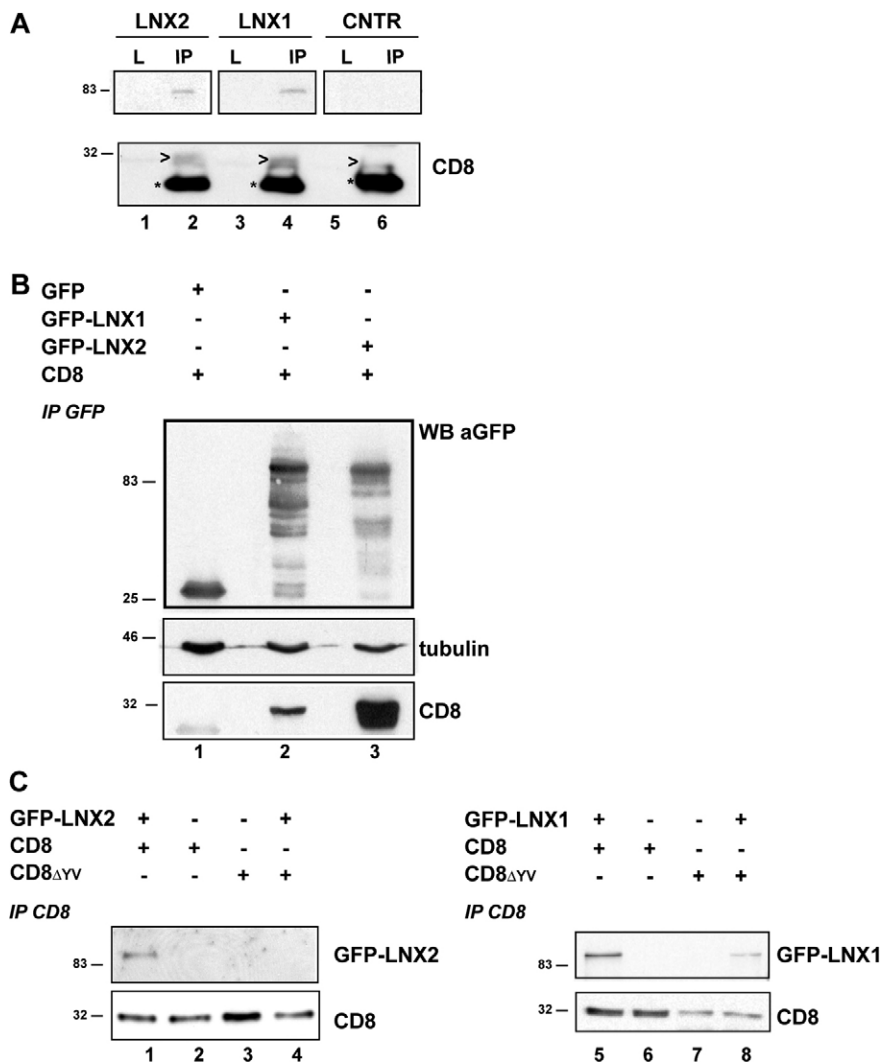


Fig. 2. In vivo interaction between CD8 α and either LNX2 or LNX1p80. (A) CD8 α was immunoprecipitated from human HPB-ALL T cells lysates and visualized by immunoblotting. A control experiment (CNTR) was also performed by using mouse IgGs. Arrowheads point to CD8 α , which was immunoprecipitated with the mouse OKT-8 antibody (lanes 2 and 4), but not with mouse IgGs (lane 6). The presence of either LNX1p80 (lane 4) or LNX2 (lane 2) in the immunoprecipitates was revealed with specific antibodies. In the control, immunoblotting was performed by concomitantly using anti-LNX1p80 and anti-LNX2 antibodies (lane 6). Aliquots (100 μ g) of total proteins were precipitated and loaded (L). However, CD8 α and LNX proteins (lanes 1, 3 and 5) were poorly detectable in these fractions. Asterisks mark the immunoglobulin light chains revealed by immunoblotting. (B) CD8 α was expressed with GFP (lane 1), GFP-LNX1p80 (lane 2) or GFP-LNX2 (lane 3) in HEK293 cells. After 24 hours of transfection, LNX proteins were immunoprecipitated from cell lysates by an anti-GFP antibody and visualized by immunoblotting. The presence of CD8 α in the immunoprecipitates was revealed with anti-CD8 α antibody. Lysates were also analysed by immunoblotting with anti-tubulin antibody as loading control. (C) CD8 α or CD8 α - Δ YV was expressed alone (lanes 2, 3, 6 and 7) or together with either GFP-LNX2 (lanes 1 and 4) or GFP-LNX1p80 (lanes 5 and 8) in HEK293 cells. After 24 hours of transfection, CD8 α was immunoprecipitated from cell lysates and visualized by immunoblotting. The presence of LNX proteins in the precipitates was revealed with an anti-LNX2 (lanes 1–4) or anti-GFP (lanes 5–8) antibody. Numbers on the left indicate molecular mass (kDa).

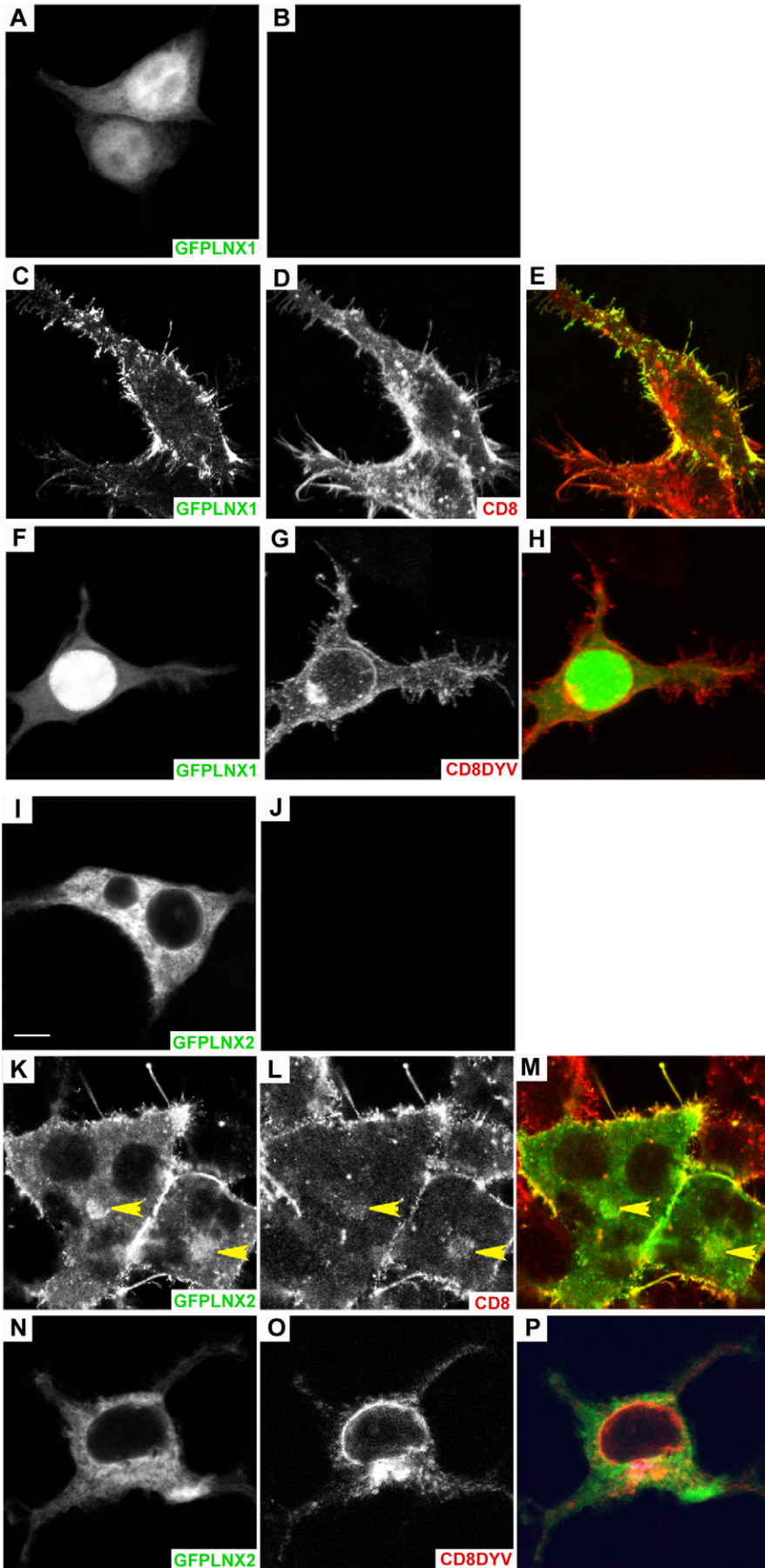


Fig. 3. CD8 α expression leads to redistribution of LNX1p80 or LNX2 to the PM. (A–P) GFP–LNX1p80 (A–H; green) or GFP–LNX2 (I–P; green) was expressed in HEK293 cells alone (A–B, I–J) or together with either CD8 α (C–E, K–M) or CD8- Δ YV (F–H, N–P). After 24 hours of transfection, cells were fixed and immunostained for CD8 α (red). (E, H, M, P) Merged images. Yellow arrowheads in K–M indicate colocalization between CD8 α and GFP–LNX2, presumably at the Golgi complex. A single confocal section is shown. Scale bars: 3 μ m.

expressed in these cells (supplementary material Fig. S1, lanes 2 and 3). Unfortunately, the low level of endogenous CD8 α protein did not allow us to co-immunoprecipitate LNX proteins from this cell fraction. Therefore, we used the human HPB-ALL T-cell line and demonstrated that, in these cells, LNX1p80 and LNX2 were pulled down by CD8 α (Fig. 2A, lanes 2 and 4). Therefore, these data suggested that such interactions also occur in the physiological context of the T-cell co-receptor CD8 α .

In order to further characterize this interaction, we decided to shift to a heterologous expression system that could be more easily handled and allow higher level of protein expression. Therefore, GFP and GFP-tagged versions of LNX1p80 and LNX2 were generated, transfected with CD8 α in HEK293 cells, and then immunoprecipitated from cell lysates by an anti-GFP antibody. The presence of CD8 α in the precipitates was tested by immunoblotting. Consistent with the previous data, GFP-LNX1p80 (Fig. 2B, lane 2) and GFP-LNX2 (Fig. 2B, lane 3), but not GFP (Fig. 2B, lane 1), were able to co-immunoprecipitate CD8 α . Likewise, CD8 α pulled-down GFP-LNX2 (Fig. 2C, lane 1) and GFP-LNX1p80 (Fig. 2C, lane 5). In the absence of CD8 α C-TVM, LNX2 interaction was completely lost (Fig. 2C, lane 4), whereas LNX1p80 interaction decreased (Fig. 2C, lane 8). Therefore, CD8 α in vitro and in vivo interacts with LNX2 and LNX1p80 and these interactions require its C-TVM.

It has been previously shown that LNX1p80 interacts with the junctional proteins JAM-4 via the second PDZ domain (Kansaku et al., 2006), and that the first two PDZ domains of LNX2 are required for binding to the cell surface coxsackievirus and adenovirus receptor (CAR) (Sollerbrant et al., 2003). To identify regions of LNX1p80 and LNX2 important for CD8 α recognition, we generated a series of truncation mutants containing separately the distinct domains, and tested their ability to bind the CD8 α cytosolic C-terminal tail or its Δ YV mutant fused to the GST (supplementary material Fig. S2). Consistent with the results of the two-hybrid assay, the N-terminal fragment of LNX2 including the RING domain, NPAY signal and the first two PDZ domains was able, although to a less extent, to interact with the CD8 α tail in a YV-dependent manner. However, neither the RING domain nor the NPAY motif alone interacted in the same way as the full-length protein. By contrast, we found that the fragment including all four PDZ domains was indispensable for a proper interaction with CD8 α . Indeed, the in vitro binding efficiency of the fragment was comparable to that observed for the full-length protein (supplementary material Fig. S2). Moreover, when expressed in HEK293 cells, it was co-immunoprecipitated by CD8 α (supplementary material Fig. S3A, lane 3). Among the four PDZ domains of LNX2, the first two appeared to have a major role because they were sufficient, although to a lesser extent, to bind the CD8 α tail in a YV-dependent manner. Similar results were overall observed for the LNX1p80 protein (supplementary material Figs S2 and S3A), thus fully confirming the observation that LNX1p80 and LNX2 interact with CD8 α via their four PDZ domains.

CD8 α expression leads to redistribution of LNX1p80 or LNX2 to the plasma membrane

To examine the intracellular localization of LNX1p80 and LNX2 in mammalian cells, GFP- or haemagglutinin (HA)-tagged constructs of the two proteins were generated and expressed in HEK293 cells. GFP-LNX1p80 exhibited both a cytosolic and nuclear localization (Fig. 3A), as previously described (Zheng et al., 2010). By contrast,

GFP-LNX2 was exclusively cytosolic (Fig. 3I). Remarkably, we found that this localization was changed upon simultaneous expression of wild-type CD8 α : both GFP-LNX1p80 (Fig. 3C–E) and GFP-LNX2 (Fig. 3K–M) appeared to redistribute at the PM, where CD8 α localized as well. In addition, GFP-LNX2 was also recruited by CD8 α at the Golgi complex (Fig. 3K–M, arrowheads). Interestingly, HA-tagged fragments containing the four PDZ domains of either LNX1p80 or LNX2 were also relocated at the PM upon CD8 α coexpression (supplementary material Fig. S3Ba–c, Bg–i). Moreover, as expected, expression of the CD8- Δ YV did not have any effect on the intracellular distribution of all these chimeric proteins (Fig. 3F–H, N–P, and supplementary material Fig. S3Bd–f, Bj–l). These results confirmed that CD8 α interacts in live cells with LNX1p80 and LNX2, strongly suggesting a functional role of these interactions.

LNX1p80 and LNX2 promote ubiquitylation of CD8 α

Next, we examined whether LNX1p80 was able to ubiquitylate CD8 α , as previously described for claudin (Takahashi et al., 2009), and whether also LNX2 was endowed of ubiquitylation activity. For this purpose, we coexpressed CD8 α , HA-tagged ubiquitin, and GFP, GFP-LNX1p80 or GFP-LNX2 in HEK293 cells (Fig. 4). After 24 hours of transfection, CD8 α was

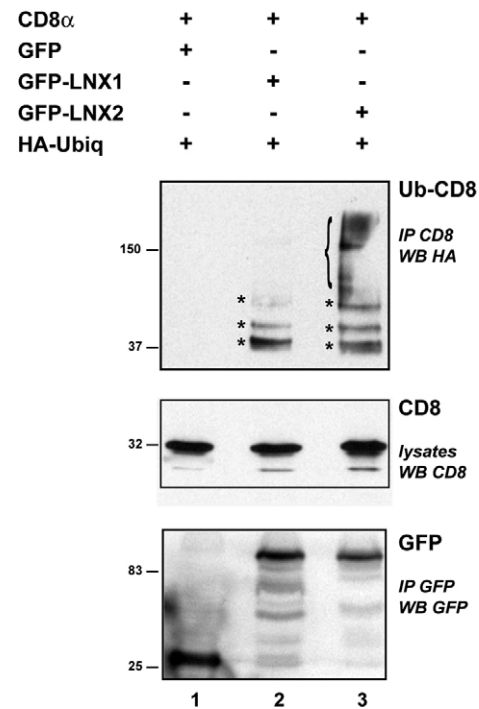


Fig. 4. LNX1p80- and LNX2-dependent ubiquitylation of CD8 α . Plasmids expressing CD8 α , HA-tagged ubiquitin and GFP, GFP-LNX1p80 or GFP-LNX2 were transiently co-transfected in HEK293 cells. After 24 hours, CD8 α was immunoprecipitated from cell lysates and the ubiquitylated CD8 α fraction (Ub-CD8) was revealed by immunoblotting with anti-HA antibody. Total lysates were also analyzed by immunoblotting with anti-CD8 α antibody. GFP-tagged LNX proteins were immunoprecipitated from cell lysates with a GFP monoclonal antibody followed by immunoblotting with an anti-GFP polyclonal antibody. The asterisks indicate three ubiquitylated CD8 α bands, which might correspond to monoubiquitylation of single, double and triple Lys residues. The parenthesis indicates the smear pattern and higher molecular weight ubiquitylated CD8 α forms detected only in the presence of LNX2. Numbers on the left indicate molecular mass (kDa).

immunoprecipitated from cell lysates and its level of ubiquitylation was analyzed by immunoblotting with an anti-HA antibody (Fig. 4). In contrast to the control (Fig. 4, lane 1), a

pattern of HA signal was found in the presence of the coexpressed GFP-LNX1p80 (Fig. 4, lane 2) or GFP-LNX2 (Fig. 4, lane 3). Because the cytosolic tail of CD8 α has three

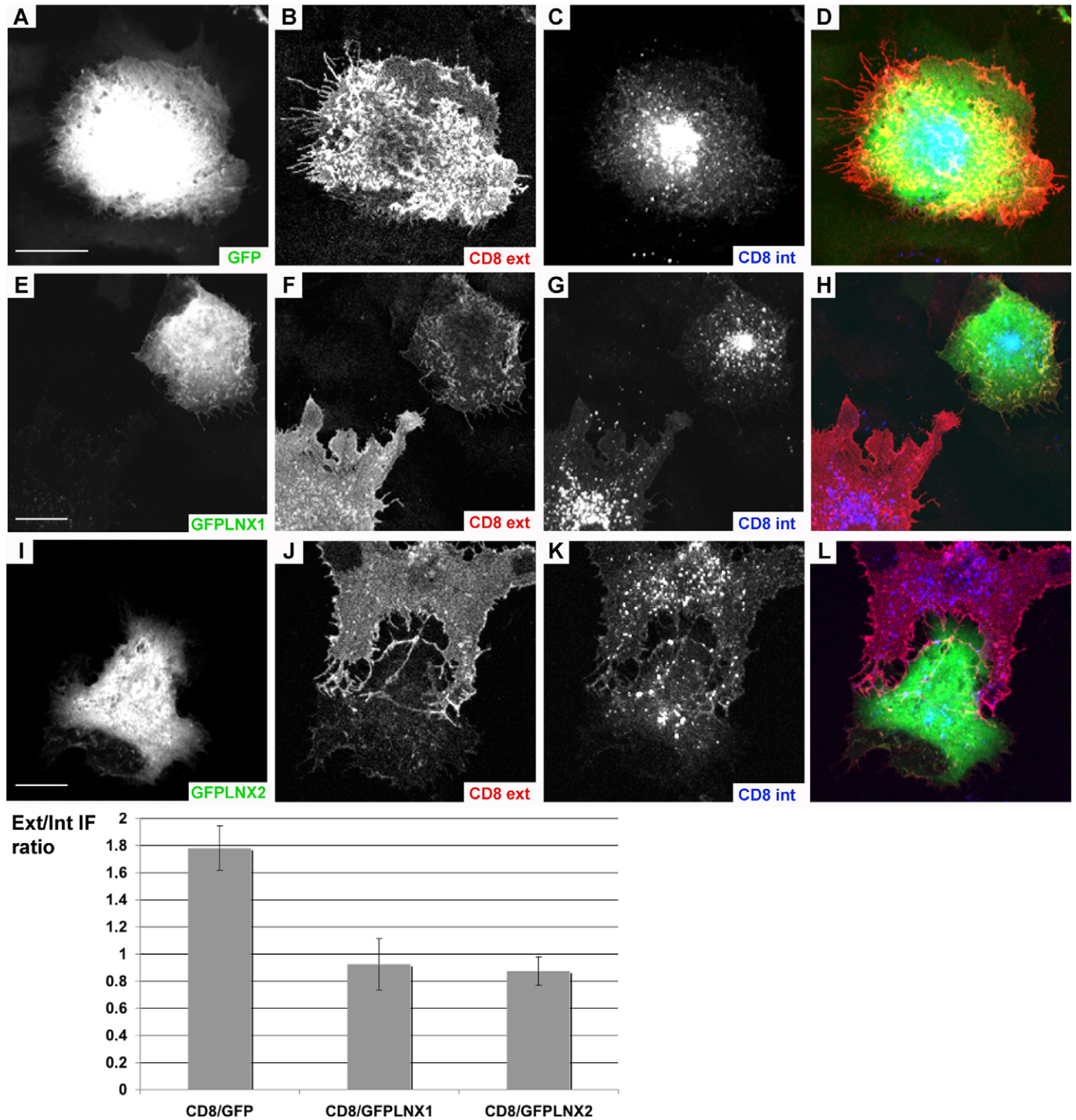


Fig. 5. High expression of GFP-LNX1p80 and GFP-LNX2, but not GFP, induces CD8 α downregulation from the PM. (A–L) Huh-7 cells expressing, by the new transfection procedure, CD8 α and a high level of GFP (A–D), GFP-LNX1p80 (E–H) or GFP-LNX2 (I–L) were fixed and analyzed by indirect immunofluorescence. (B,F,J) Cells stained for CD8 α before permeabilization in order to label the CD8 α fraction localized on the PM (CD8 ext; red). (C,G,K) Cells stained for CD8 α after permeabilization to label the CD8 α intracellular fraction (CD8 int; blue). (D,H,L) Merged images. Expression of GFP (A; green), GFP-LNX1p80 (E; green) or GFP-LNX2 (I; green) are shown. A single confocal section is shown. Histogram shows the ratio between the external and the internal CD8 α fraction quantified for the three distinct co-transfections, as described in Materials and Methods. Scale bars: 3 μ m.

potential Lys residues for ubiquitylation (Fig. 1A), the lower three bands visualized by the anti-HA antibody (Fig. 4, asterisks) might correspond to monoubiquitylation of single, double and triple Lys residues. Higher bands, possibly corresponding to polyubiquitylated forms, and a smear pattern were detected exclusively when CD8 α was coexpressed with LNX2 (Fig. 4, lane 3). These results clearly indicated that both LNX1p80 and LNX2 induce CD8 α ubiquitylation.

LNX1p80 and LNX2 control CD8 α localization at the plasma membrane

While performing the immunofluorescence experiments on HEK293 cells, we observed a reduction in CD8 α levels at the PM, which correlated with higher levels of LNX protein

expression (data not shown). In order to verify and clearly visualize this effect, we decided to shift to the human hepatoma cell line Huh-7, because these cells are bigger and more spread than HEK293 cells, allowing a better visualization of protein localization at the PM. Moreover, we changed our transfection procedure by initially transfecting cells with GFP–LNX1p80, GFP–LNX2 or GFP and, only 24 hours later, with CD8 α . This allowed us to accumulate high level of LNX proteins in cells before CD8 α expression. Then, after a further 24 hours, cells were fixed and, before detergent permeabilization, directly treated with an anti-CD8 polyclonal antibody to uniquely label the CD8 α fraction localized at the PM. Cells were then permeabilized and the CD8 α intracellular fraction was revealed by using an anti-CD8 monoclonal antibody. As shown in Fig. 5,

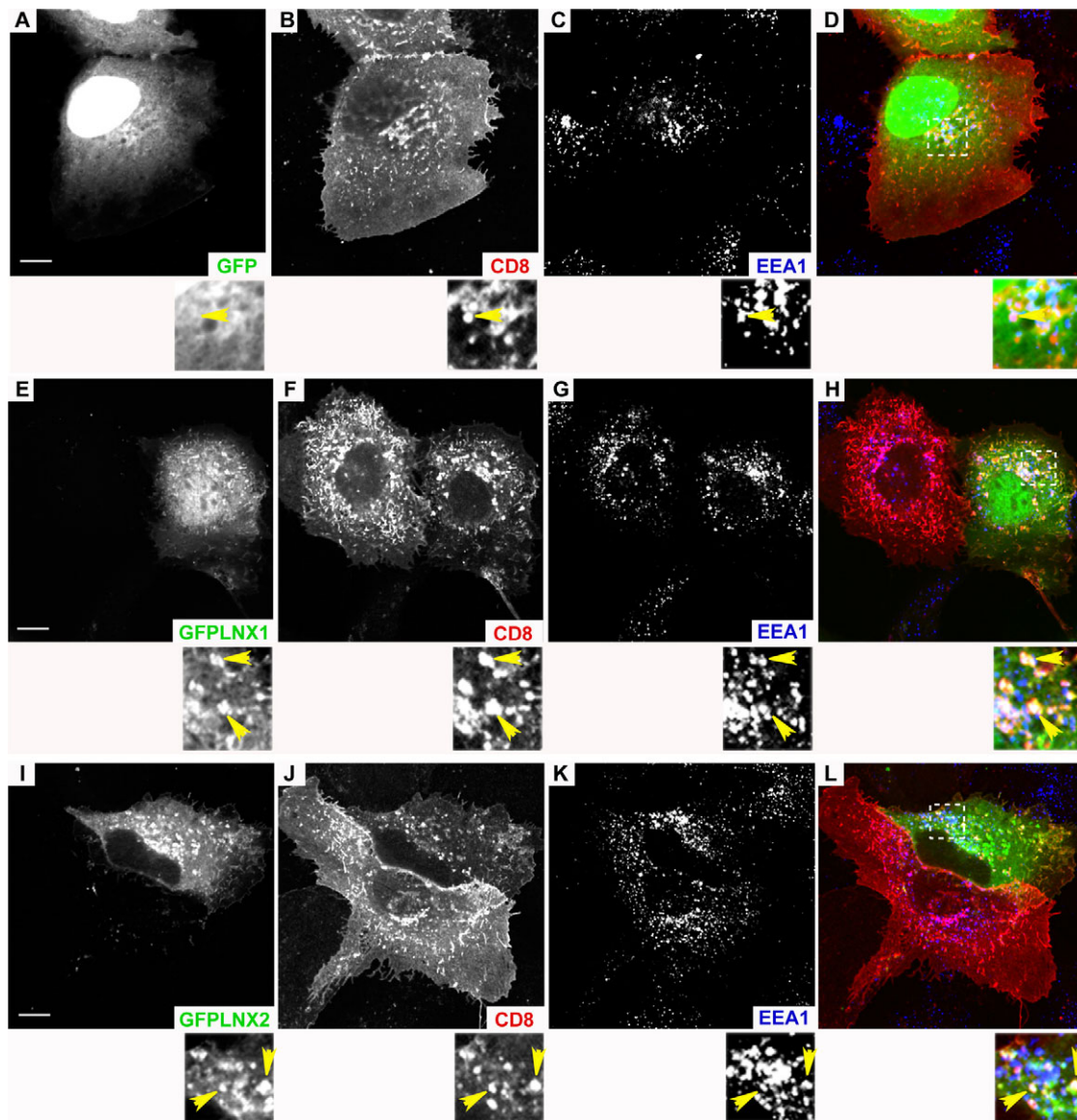


Fig. 6. High expression of GFP–LNX1p80 and GFP–LNX2, but not GFP, promotes CD8 α transport to the early endosomes. (A–L) Huh-7 cells expressing, by the new transfection procedure, CD8 α and a high level of GFP (A–D), GFP–LNX1p80 (E–H) or GFP–LNX2 (I–L) were fixed and stained for CD8 α (B,F,J; red) and the early endosomes marker EEA1 (C,G,K; blue). (D,H,L) Merge images. Higher magnification of the cell areas marked by yellow lines are shown below. Arrowheads indicate endocytic structures where CD8 α colocalizes with GFP–LNX1p80 or GFP–LNX2, but not GFP. Asterisks mark cells expressing only CD8 α . A single confocal section is shown. Scale bars: 3 μ m.

upon coexpression with either GFP–LNX1p80 (Fig. 5E–H) or GFP–LNX2 (Fig. 5I–L), the amount of CD8 α localized at the PM was strongly decreased in comparison with cells expressing CD8 α alone (Fig. 5F,J) or together with GFP (Fig. 5A–D). We quantified this decrease by measuring the ratio between the PM and intracellular signal detected by immunofluorescence (for quantification see Materials and Methods). As shown in Fig. 5, with respect to the control coexpression of CD8 α and GFP, only ~50% of CD8 α at the PM was detected upon GFP–LNX1p80 or GFP–LNX2 coexpression. Therefore, we concluded that LNX1p80 and LNX2 modulate CD8 α protein levels at the PM.

LNX1p80 and LNX2 are involved in endocytic trafficking and degradation of CD8 α

The monoubiquitylation of CD8 α and its downregulation from the PM in the presence of LNX1p80 and LNX2 suggest that the two

LNX proteins are involved in CD8 α internalization and degradation. To address this hypothesis, Huh-7 cells expressing (by the new transfection procedure) a high level of GFP–LNX1p80, GFP–LNX2 or GFP together with CD8 α were examined by immunofluorescence using antibodies specific for an early endosomes marker (EEA1) and a lysosomal marker (LAMP1), aiming to detect accumulation of CD8 α into endocytic compartments. We found that in the presence of either GFP–LNX1p80 (Fig. 6E–H; Fig. 7E–H) or GFP–LNX2 (Fig. 6I–L; Fig. 7I–L), concomitantly to its downregulation from the PM, a fraction of CD8 α accumulated in EEA1- or LAMP1-positive compartments (Fig. 6E–L; Fig. 7E–L, arrowheads). By contrast, in cells expressing only CD8 α (Fig. 6F,J, asterisks) or CD8 α together with GFP (Fig. 6A–D; Fig. 7A–D), CD8 α was not significantly detected in endocytic structures and was mainly visualized at the PM. Similar results were also observed in HEK293 cells (data not shown).

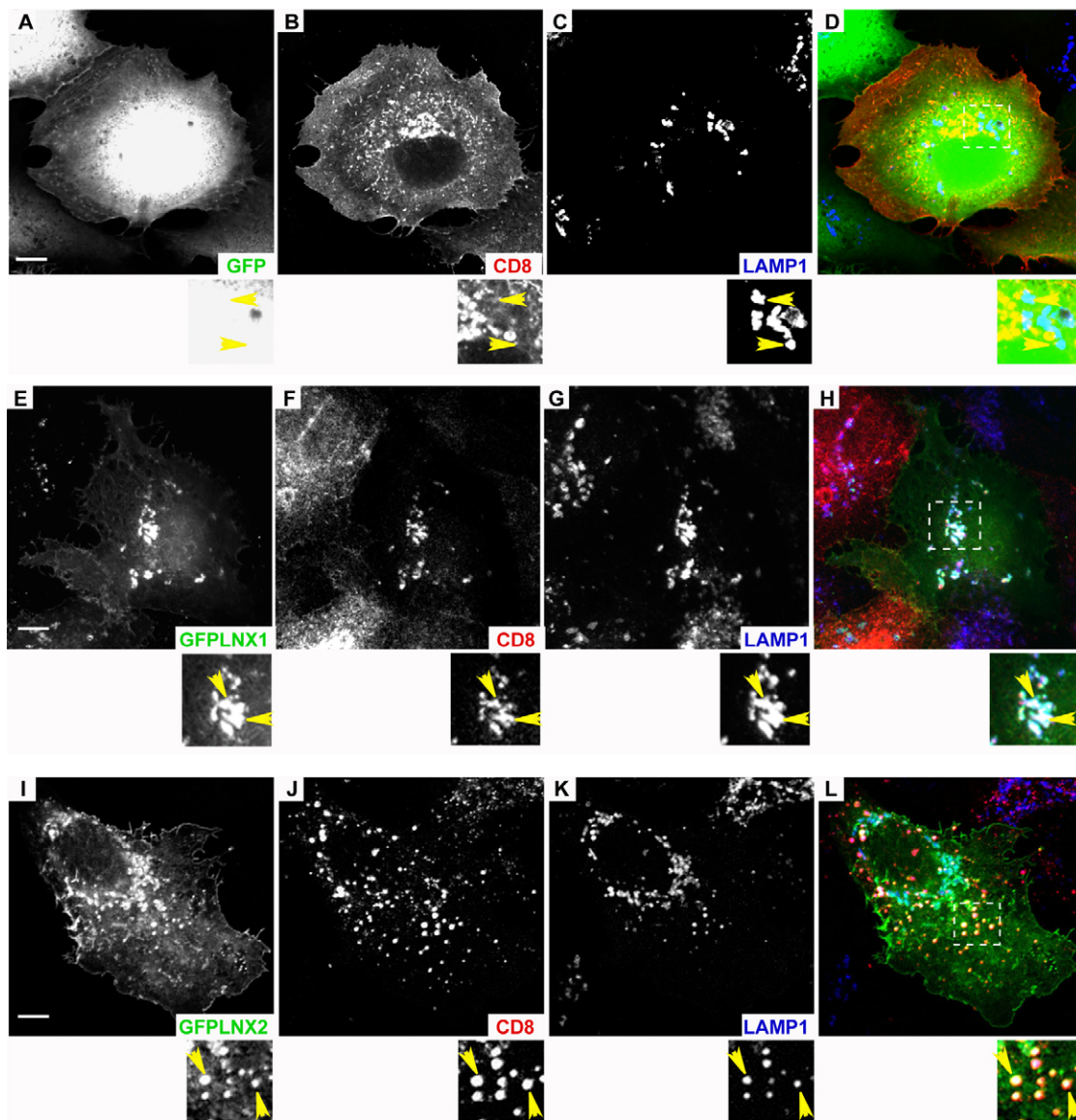


Fig. 7. High expression of GFP–LNX1p80 and GFP–LNX2, but not GFP, promotes CD8 α transport to the lysosomes. (A–L) Huh-7 cells expressing, by the new transfection procedure, CD8 α and a high level of GFP (A–D), GFP–LNX1p80 (E–H) or GFP–LNX2 (I–L) were fixed and stained for CD8 α (B,F,J; red) and the lysosomal marker LAMP1 (C,G,K; blue). (D,H,L) Merged images. Higher magnification of the cell areas marked by yellow lines are shown below. Arrowheads indicate endocytic structures where CD8 α colocalizes with GFP–LNX1p80 or GFP–LNX2, but not GFP. A single confocal section is shown. Scale bars: 3 μ m.

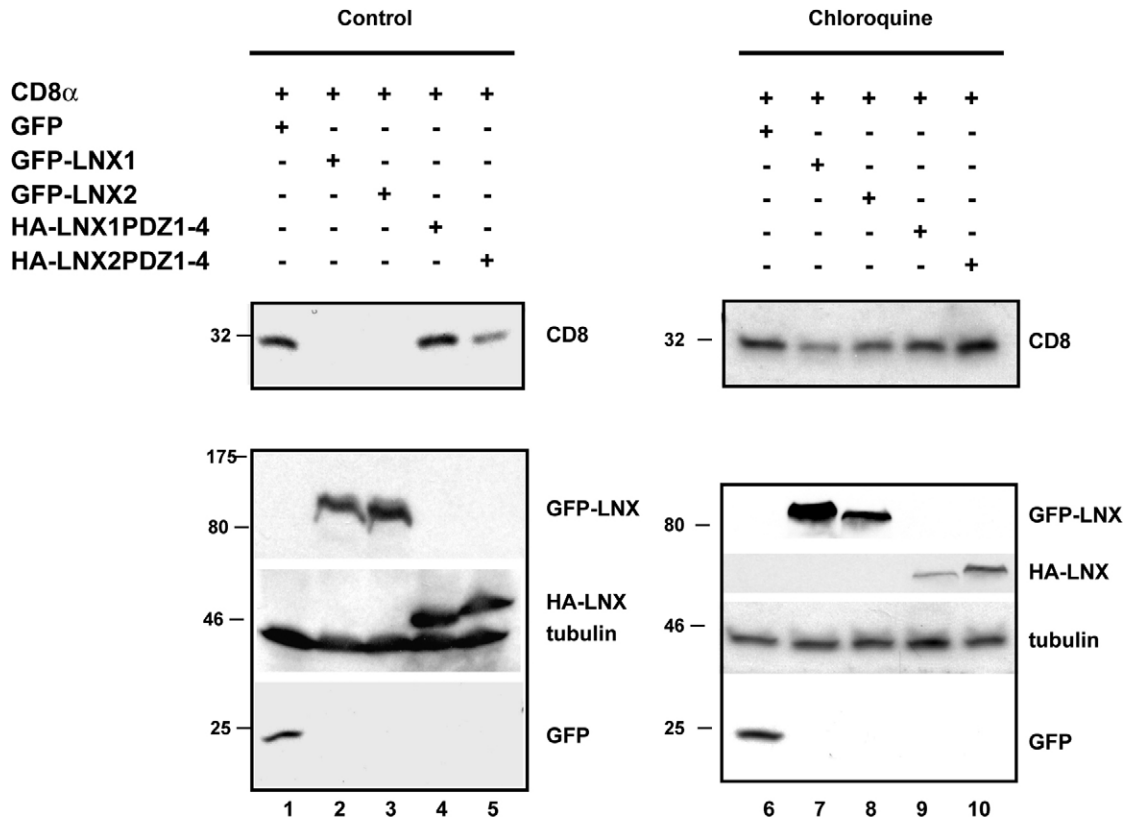


Fig. 8. Lysosomal degradation of CD8 α protein in cells highly expressing either GFP-LNX1p80 or GFP-LNX2. HEK293 cells expressing, by the new transfection procedure, CD8 α and a high level of GFP (lanes 1 and 6), GFP-LNX1p80 (lanes 2 and 7), GFP-LNX2 (lanes 3 and 8), HA-tagged PDZ1–PDZ4 domains of LNX1p80 (lanes 4 and 9) or LNX2 (lanes 5 and 10), were cultured with or without chloroquine treatment. After cell lysis, CD8 α protein was immunoprecipitated from equal cell lysate aliquots and revealed by immunoblotting. In parallel, equal cell lysate aliquots were used to reveal GFP and GFP-tagged LNX proteins with an anti-GFP monoclonal antibody, and HA-tagged PDZ domains of LNX1p80 and LNX2 with an anti-HA monoclonal antibody. Lysates were also analyzed by immunoblotting with anti-tubulin antibody as loading control. Numbers on the left indicate molecular mass (kDa).

Next, we examined whether CD8 α downregulation from the PM observed by the new transfection procedure resulted in co-receptor degradation. We have previously observed that simultaneous co-transfection of CD8 α and GFP-LNX proteins induced CD8 α ubiquitylation but did not decrease the total amount of CD8 α in the whole lysate (Fig. 4, lanes 1–3). By contrast, Fig. 8 clearly shows that, on equal conditions of co-transfection efficiency and input protein level (tubulin protein level), when GFP-LNX1p80 (Fig. 8, lane 2) or GFP-LNX2 (Fig. 8, lane 3) are highly expressed, the total amount of CD8 α in the whole lysate was smaller than that of GFP-coexpressing cells (Fig. 8, lane 1). Remarkably, such effect on CD8 α was not observed when CD8 α was coexpressed with the HA-tagged PDZ domains of either LNX1p80 or LNX2 (Fig. 8, lanes 4 and 5), which bind CD8 α (supplementary material Figs S2 and S3), but lack the RING domains. To understand whether this degradation takes place in the lysosomes, we treated cells with the lysosome inhibitor chloroquine (Fig. 8, lanes 6–10) and found that the amount of CD8 α in the whole lysate was significantly increased by this treatment (Fig. 8, lanes 7 and 8). These data strongly suggest that LNX1p80 and LNX2 induce CD8 α transport to the lysosomes and subsequent degradation.

Discussion

In this paper, we identify and characterize the interaction between the human co-receptor CD8 α and LNX1p80 and LNX2 proteins.

Our data show that this binding occurs *in vitro* and *in vivo* and mutually affects the localization of each partner: CD8 α recruits LNX1p80 or LNX2 from the cytosol to the PM, whereas, remarkably, LNX1p80 or LNX2 expression leads to CD8 α ubiquitylation, downregulation from the PM, endocytosis and degradation.

RING-based E3 ligases confer specificity to ubiquitylation by recognizing target substrates. Accordingly, they have been linked to the control of many cellular processes and to multiple human diseases. However, despite their relevance, the functional characterization of most of them remains at a rudimentary stage (Deshaies and Joazeiro, 2009). The activity of LNX1 as E3 ubiquitin ligase has been recently explored. Two splicing variants of LNX1 exist, p70 and p80. The LNX1p70 binds the junctional protein JAM4, and its overexpression facilitates JAM4 endocytosis (Kansaku et al., 2006). On the other hand, LNX1p80 is an interacting partner of claudin-1 and promotes its ubiquitylation, removal from the tight junctions and transport to the lysosomes (Takahashi et al., 2009). Interestingly, LNX1 downregulation has been associated to gliomas (Chen et al., 2005) and to the cardiovascular Kawasaki infectious disease (Burgner et al., 2009), indicating its indispensable relevance for the proper functionality of distinct tissues and organs. In contrast to LNX1, LNX2 has been poorly studied and the only interacting protein so far identified was the cell surface coxsackievirus and adenovirus receptor (CAR)

(Mirza et al., 2006). No information is available to date on whether LNX2 behaves, similarly to LNX1, as an E3 ubiquitin ligase in mammalian cells. Here, we shed much light on the function of both LNX proteins: we demonstrate that they are expressed in human T lymphocytes and we identify an important novel partner for them, the human TCR co-receptor CD8 α . This finding suggests that LNX proteins play a role in the regulation of T-cell tolerance and immunity, similarly to the E3 ligase c-Cbl, which also has a RING finger domain (Jeon et al., 2004; Naramura et al., 2002). Future work will test this interesting hypothesis.

Although the anterograde transport of CD8 α and β chains along the exocytic pathway has been described (D'Angelo et al., 2009; Erra et al., 1999; Goldrath et al., 1997; Pascale et al., 1992), whether and how CD8 localization at PM is regulated by endocytic trafficking and which signals and cellular machineries are involved are so far largely unknown. The identification of the LNX ubiquitin ligases as interacting proteins of CD8 α , their redistribution at the PM upon CD8 α expression, and the effect of LNX protein expression on CD8 α localization and expression can help answer these questions and help in understanding how impairment of CD8 function and localization at the PM might lead to immune diseases. It will also be interesting to investigate whether and how LNX proteins regulate the localization of the CD8 $\alpha\beta$ heterodimer.

Despite the strong similarity between LNX1p80 and LNX2 structure and function, potentially interesting differences might distinguish them. In contrast to LNX1p80, LNX2 is recruited by CD8 α also to the Golgi complex; its interaction is more severely dependent on the C-TVM of CD8 α , and LNX2 expression also induces a smear pattern of ubiquitylation, which might correspond to CD8 α polyubiquitylated forms. Intriguingly, although mono- or multimono-ubiquitylation has been notably involved in endocytosis (Mukhopadhyay and Riezman, 2007), polyubiquitylation has also been associated with direct sorting from the Golgi to the vacuole of the yeast protein Gap1 (Soetens et al., 2001). Therefore, LNX2 might be also involved in the regulation of CD8 α transport to, through, or from the Golgi complex, which has already been shown to be strongly dependent on the C-TVM of the co-receptor (D'Angelo et al., 2009). Further work is required to verify this hypothesis.

In conclusion, our results identify two major interacting partners of the human co-receptor CD8 α , providing the first link between the possible regulation of the function of CD8 α at the PM of lymphocytes and ubiquitylation, endocytosis and lysosomal degradation.

Materials and Methods

Reagents

All of the culture reagents were obtained from Sigma-Aldrich (Milan, Italy). The solid chemical and liquid reagents were obtained from E. Merck (Darmstadt, Germany), Farmitalia Carlo Erba (Milan, Italy), Serva Feinbiochemica (Heidelberg, Germany), Delchimica (Naples, Italy) and BDH (Poole, United Kingdom). The radiochemicals were obtained from Perkin Elmer (Bruxelles, Belgium). Protein A-Sepharose CL-4B and the enhanced chemiluminescence reagents were from Amersham Biosciences (Milan, Italy).

Antibodies

The following antibodies were used: the OKT8 mouse anti-CD8 α monoclonal antibody from Ortho (Raritan, NJ); the N1 mouse anti-CD8 α monoclonal antibody and rabbit anti-CD8 α polyclonal antibody from M. Jackson (Martire et al., 1996); rabbit anti-LNX1 antibody (Kansaku et al., 2006); rabbit and mouse anti-GFP and anti-HA antibodies (Santa Cruz Biotechnology); mouse anti-EEA1 monoclonal antibody (BD Transduction Laboratories, Lexington, KY); mouse anti-CD7107a (LAMP1) monoclonal antibody (Biologend, San Diego, CA); peroxidase-conjugated anti-mouse and anti-rabbit IgG (Sigma-Aldrich, Milan, Italy); and

Texas-Red-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA).

Antibody production

A polyclonal antiserum against LNX2 was generated by immunizing rabbits with GST fused to the first 50 amino acids of LNX2, which are specific for LNX2 and not conserved in LNX1p80. The antibody was affinity-purified as described (Nielsen et al., 2000) and its ability to recognize LNX2, but not LNX1p80, was verified by western blot analysis of extracts from cells transfected either with GFP-LNX1p80 or GFP-LNX2.

Yeast two-hybrid assay

In order to construct the bait vector, the cDNA fragment encoding the wild-type or Δ TY mutant C-terminal tail of CD8 α was amplified by PCR and subcloned in the vector pGBKT7 (Clontech). The vector expressing the wild-type CD8 α C-terminal tail was used to screen a human liver cDNA library made in the pGAD10 vector (Clontech) using the *Saccharomyces cerevisiae* strain AH109. The transformants were plated on synthetic medium lacking histidine, leucine, and tryptophan. His⁺ colonies were transferred onto nitrocellulose filters and assayed for β -galactosidase activity. His⁺ and *lacZ*⁺ colonies were indicative of positive interaction. cDNA from positive clones was extracted, purified and sequenced. The recovered library plasmids were then tested for interaction with CD8 α tail by co-transformation with the vectors pGBKT7, pGBKT7-CD8 α or pGBKT7-CD8 α - Δ TY in *S. cerevisiae* strain AH109. To quantify the interactions, the β -galactosidase assay was again used.

cDNA cloning and plasmid construction

The expression vector FLTR β T8 for wild-type CD8 α and CD8 α - Δ YV (Iodice et al., 2001; Mottola et al., 2000) and HA-ubiquitin (Mauro et al., 2006) have been previously described. cDNAs encoding LNX1p80 (ID 4995278) and LNX2 (ID 5541168) were obtained from I.M.A.G.E. Consortium. GFP-LNX1p80 and GFP-LNX2 expression vectors were generated by PCR and subcloning of *LNX1p80* and *LNX2* cDNAs in the pEGFP-N1 vector (Clontech).

For in vitro binding assays, GST fused to wild-type or Δ YV-mutant CD8 α were expressed using pGEX-6P-1 (Pharmacia Biotech). LNX1p80, LNX2 and all their truncated forms were subcloned in the expression vector pcDNA3 (Clontech). The following *LNX1p80* cDNA fragments were expressed for the in vitro binding assay shown in supplementary material Fig. S1: RING-NPAF (1–760 bp); RING (1–309 bp); NPAF (309–760 bp); PDZ(1–4) (760–2187 bp); PDZ(1–2) (760–1415 bp); PDZ(3–4) (1480–2187 bp); PDZ(1) (760–1074 bp), PDZ(2) (1114–1415 bp).

The following *LNX2* cDNA fragments were expressed for the in vitro binding assay shown in supplementary material Fig. S1: RING-NPAY (1–666 bp); RING (1–340 bp); NPAY (510–666 bp); PDZ(1–4) (637–2073 bp); PDZ(1–2) (682–1386 bp); PDZ(3–4) (1378–2073 bp); PDZ(1) (682–983 bp), PDZ(2) (961–1386 bp).

In vitro binding assay

LNX1p80 or LNX2 proteins were translated in vitro and labelled with ³⁵S-methionine using the TnT^R coupled reticulocyte lysate system (Promega). 10 μ l of the full-length LNX proteins or fragments were incubated with 30 μ l of glutathione-Sepharose beads loaded with GST or GST fused to wild-type or Δ YV-mutant CD8 α . Bound proteins were eluted from the beads and analysed by SDS-PAGE and autoradiography. In all the GST-pull down experiments, the loading control (L) represents 20% of the whole amount of in vitro translated proteins used for incubation.

Cell culture, transfection and immunofluorescence

Human embryonal kidney HEK293 and human hepatoma Huh-7 cells were routinely grown at 37°C in Dulbecco's modified essential medium (DMEM), containing 10% foetal bovine serum (FBS). HPB-ALL T cells were cultured in RPMI 1640, containing 15% FBS.

HEK293 and Huh-7 cells were transfected by using FuGene 6.0 (Roche) according to the manufacturer's instructions. The effect of CD8 α expression on LNX protein localization was obtained by simultaneous co-transfection of CD8 α and LNX protein expression vectors and, 24 hours later, cell fixation or lysis. To observe the effect of LNX protein expression on CD8 α localization and protein level, cells were initially transfected with LNX protein expression vectors in order to allow accumulation of LNX proteins and, 24 hours later, transfected a second time with CD8 α expression vectors. Then, after a further 24 hours, they were either lysed or fixed.

Indirect immunofluorescence was performed as previously described (Mottola et al., 2000). Single confocal images were acquired at 63 \times and 100 \times magnification on a Meta Zeiss Confocal Microscope (Carl Zeiss, Jena, Germany).

To separately stain CD8 α protein on either cell surface or intracellular membranes, cells were incubated after fixation with the rabbit anti-CD8 α polyclonal antibody, then permeabilized and incubated with the mouse anti-CD8 α

monoclonal antibody (Mottola et al., 2000). Two distinct secondary antibodies allow separately visualizing the two fractions. Then, in order to measure the ratio between levels of PM and intracellular CD8 α , the immunofluorescence intensity in the two channels was measured by using Adobe Photoshop and NIH ImageJ Biophotonic programs. For each co-transfection, 15 cells were considered for quantification. The results are given as mean \pm s.d.

Preparation of cell extracts, immunoprecipitation SDS-PAGE and western immunoblotting

Cells were lysed with 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA pH 8.0, and 1% Triton X-100. For all the experiments on transfected HEK293, 6×10^6 cells were lysed and used for each co-immunoprecipitation. For the experiment on HPB-ALL cells, 100×10^6 cells were used for each co-immunoprecipitation. Immunoprecipitation was performed by overnight incubation with OKT-8, a conformation-sensitive antibody anti-CD8 α , followed by addition of Protein A-Sepharose beads (Pharmacia). The immunoprecipitated pellets were washed, treated for SDS-PAGE and resolved on a 10% polyacrylamide gel. For each experiment, aliquots of the lysate (100 μ g total protein per aliquot) were also precipitated with acetone and treated for SDS-PAGE. Next, proteins were transferred to nitrocellulose filters, which were then incubated with primary antibodies diluted in blocking buffer (5% non-fat dry milk, 0.1% Tween-20 in PBS), followed by peroxidase-conjugated secondary antibodies. For anti-LNX1p80 and anti-LNX2 antibodies, western blot was performed in PBS containing 1% BSA. After washing, bound antibodies were detected by enhanced chemiluminescence (Amersham Biosciences).

CD8 α degradation was detected by previously treating cells with or without 90 μ M chloroquine for 16 hours.

Preparation of human T cells

Peripheral blood mononuclear cells were isolated from leukopacks (Etablissement Français du Sang) by Ficoll gradient (MSL, Eurobio). T and NK cells were then isolated by removing monocytes with CD14⁺ columns as recommended by the manufacturer (Miltenyi Biotec).

Real time RT-PCR

Real time reverse-transcription PCR was performed as previously described (Ben Amara et al., 2010). Total RNA was extracted from the T-cell-enriched fraction and reverse transcribed using the MMLV-RT kit according to the manufacturer's protocol (Invitrogen). Forward and reverse primers were designed with the free web software Primer3.

The following primers were used: For human LNX1: forward 5'-GG-AATTACCACGGTCTGTAT-3', reverse 5'-TGTATGCTGGTTCCTTC-AAC-3'; expected PCR fragment length, 154 bp. For human LNX2: forward 5'-TCTTCATAAACGGGACTCTGGT-3', reverse 5'-GCTGCTATGATTTCTGC-TTCT-3'; expected PCR fragment length 291 bp. For human β -actin: forward 5'-GGAAATCGTGCCTGACATTA-3', reverse 5'-AGGAAGGAAGCTGGAAG-AG-3'; expected PCR fragment length: 309 bp.

Real time PCR was performed using the Applied Biosystem 7900HT Fast real-time PCR system according to the manufacturer's recommendations. The experiments were performed in triplicate.

Acknowledgements

We thank Giuliana De Luca, Luisa Iodice and Alessandro Sorrentino for participating in the initial steps of the work; Cecilia Bucci for helping to set up the two-hybrid assay and critical reading of the manuscript; A. Leonardi for generously providing the HA-ubiquitin construct; André Le Bivic for temporary hospitality in his laboratory; the group of Eric Ghigo and Jean-Louis Mege for technical support; and Massimo Mallardo for helpful discussion and critical reading of the manuscript. We hereby declare that none of the authors have a financial interest related to this work.

Funding

This work was supported by grants from Ministero Università Ricerca Scientifica e Tecnologica to S.B.; and from Telethon Italia [grant number GGP09029].

Supplementary material available online at

<http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.081224/-/DC1>

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