

Functional role of c-Jun NH₂-terminal kinase-associated leucine zipper protein (JLP) in lysosome localization and autophagy

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SUMMARY Lysosomes are involved in many cellular functions, and in turn lysosomal dysfunction underlies a variety of diseases, including cancer and neurodegenerative diseases. Lysosomes are distributed broadly in the cytoplasm and can move throughout the cell in kinesin- and dynein-dependent manners. Although many mechanisms of lysosomal transport have been reported, how lysosomal transport is regulated has yet to be fully elucidated. In this study we analyzed c-Jun NH₂-terminal kinase-associated leucine zipper protein (JLP), an adaptor of kinesin and dynein motor proteins, and found that lysosomes were localized toward the cell periphery in *JLP* knockdown cells, leading to the impairment of autophagosome-lysosome fusion. Furthermore, we performed rescue experiments using wild-type JLP and its various deletion mutants. The results indicated that JLP may regulate lysosome localization and autophagy through interaction of JLP with kinesin-1 heavy chain, but not with dynactin p150^{Glued} or lysosomal transmembrane protein 55b. Our findings provide new insights into the mechanisms of lysosomal trafficking regulation. This study contributes to the understanding of how lysosomes exert their multiple functions, potentially leading to the identification of molecular targets for diseases caused by lysosomal dysfunction.

Keywords dynein, kinesin, lysosomal transport

1. Introduction

Lysosomes are well-known to be terminal, degradative organelles. However, increasing evidence indicates that lysosomes contribute to many other cellular processes, such as antigen presentation, apoptosis, and metabolic signaling (1). Similarly, lysosomal dysfunction underlies a variety of diseases, including cancer, lysosomal storage, and neurodegenerative diseases (1-3). Lysosomes are distributed broadly throughout the cytoplasm and can move toward the microtubule plus-ends and minus-ends, as mediated by kinesin and dynein motors, respectively (1,2). Previous studies have reported many mechanisms of lysosomal transport. In both the kinesin- and dynein-dependent transport of lysosomes, the small GTPase Rab7 has been shown to play important roles in recruiting the motor proteins to the lysosomes (1,2). In addition, Willett *et al.* (4) recently proposed that the lysosomal transmembrane protein 55b (TMEM55B) participates in the regulation of dynein-dependent transport of lysosomes through interaction with the motor adaptor c-Jun NH₂-terminal kinase-associated leucine zipper protein (JLP).

JLP (also known as SPAG9 or JIP4) was first identified as a scaffold protein in the mammalian mitogen-activated protein kinase (MAPK) signaling pathway (5,6), and subsequently was found to also function as an adaptor protein that links cargoes to kinesin and/or dynein motors (7-9). Recent studies have shown that JLP is a multifunctional protein involved in axonal transport, cytokinesis, and oxidative stress-induced cell death (10-14). In this study, we explored the role of JLP in lysosome localization and autophagy, and propose a novel, TMEM55B-independent mechanism of intracellular lysosomal trafficking.

2. Materials and Methods

2.1. Cell culture and reagents

U87, HeLa, HT1080, and HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Wako, Tokyo, Japan) as described previously (13,14). Amino acid-free DMEM was obtained from Wako.

2.2. Plasmids and viral vector preparation

The pLVTH lentivirus plasmid vectors for two short hairpin RNAs (shRNAs) against human JLP, shJLP#1 and shJLP#2, as well as for scramble shRNA (shScr), were described previously (14). The JLP deletion mutants, including those that lacking the dynactin p150^{Glued}-binding domain (DBD), C-terminal and N-terminal half regions of JLP [amino acid residues 371-490, 657-1,321, and 1-646 of human JLP, respectively (RefSeq accession number NP_00112400)] were generated using overlapping PCR as described by Ito *et al.* (15). The JLP deletion mutants were expressed as hemagglutinin (HA)-tagged proteins using the pCL20c-CMV lentivirus plasmid vector (16). The pCL20c-CMV expression vectors for tandem fluorescent-tagged LC3, HA-tagged wild-type JLP, and JLP mutants lacking MAPK-binding domain (MBD) or kinesin-1 heavy chain (KHC)-binding domain (KBD) were previously described (10,14). To express the HA-JLP proteins at suboptimal levels in cells, a 405-bp deletion was made in the CMV promoter/enhancer region based on Morita *et al.* (17), referred to as CMV Δ 2. However, in some experiments, the original CMV promoter/enhancer was used (see Figure 3 legend). Flag-tagged, truncated TMEM55B [amino acid residues 2-207 (RefSeq accession number NP_653169)], containing a large cytosolic N-terminal domain (CD) of human TMEM55B, was expressed using the pCL20c-CMV vector. All PCR products were verified by sequencing. Lentiviral vectors were produced as previously described (16).

2.3. Immunoprecipitation and western blot analysis

To analyze protein-protein interactions, immunoprecipitation-western blot analysis was performed as previously described (18). Briefly, after transient co-expression of HA-tagged and Flag-tagged proteins, cells were lysed in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P40, 0.25% sodium deoxycholate, 1 mM EDTA) containing Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO, USA), immunoprecipitated with anti-HA antibody beads (Wako), and analyzed by western blotting using a horseradish peroxidase-conjugated anti-Flag antibody (Sigma-Aldrich). Total cell lysates were prepared and analyzed by western blotting as described previously (16), using anti-actin (1:2,000; A6050; Sigma-Aldrich), anti-p150^{Glued} (1:1,000; 610474; BD Biosciences, San Jose, CA, USA), anti-LC3 (1:2000; MBL, Nagoya, Japan), and anti-JLP (0.25 μ g/mL (13)) primary antibodies.

2.4. Immunocytochemistry, fluorescence, and quantification

Immunocytochemistry was carried out as previously described (16), using anti-HA (1:100; 11867423001, Sigma-Aldrich) and anti-LAMP-1 (1:300; H4A3, Developmental Studies Hybridoma Bank, Iowa City,

IA, USA) primary antibodies. The secondary antibodies were Alexa Fluor 568- and 647-conjugated goat, anti-mouse IgG (both 1:500; Thermo Fisher Scientific, Waltham, MA, USA). Fluorescent images were captured using confocal laser scanning microscopes [(LSM510 META, Carl Zeiss, Oberkochen, Germany, Figures 1-4 and Figure S1, <http://www.ddtjournal.com/action/getSupplementalData.php?ID=53>); (TCS SP8; Leica, Wetzlar, Germany, Figure 5); and (BZ-9000, Keyence, Osaka, Japan, Figure S2, <http://www.ddtjournal.com/action/getSupplementalData.php?ID=53>)]. Quantitative analyses of lysosome distribution in Figures 1-4 and colocalization in Figure 5 were performed essentially as described by Willett *et al.* (4) and Michelet *et al.* (19), respectively.

2.5. Statistical analysis

Significance was determined using a two-tailed, unpaired Student's *t*-test. Values of $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. JLP knockdown results in altered lysosome localization

We first examined whether JLP is involved in lysosome localization. To this end, we generated JLP knockdown cells using two lentiviral shRNAs against JLP (shJLP#1 and shJLP#2). As shown in Figure 1A, highly reduced levels of JLP protein were observed in U87 and HeLa cells expressing shJLP#1 or shJLP#2, as compared to their respective parent cells and control cells expressing shScr. We then performed immunostaining with an antibody against LAMP-1, a lysosome marker, and observed an altered distribution of lysosomes in the JLP knockdown cells. Lysosomes were dispersed toward the cell periphery in the JLP knockdown cells, as compared to parent and control shScr-expressing cells (Figures 1B and 1C). A similar result was obtained when we analyzed HT1080 cells in a similar manner (Figure S1, <http://www.ddtjournal.com/action/getSupplementalData.php?ID=53>). These results strongly suggest that JLP plays a role in lysosome localization, and that JLP knockdown causes lysosome distribution to the cell periphery.

3.2. JLP is involved in lysosome localization independently from its interaction with dynactin p150^{Glued}

As lysosomes are known to move along microtubules toward the centrosome *via* the dynein motor (1,2), we hypothesized that the altered lysosomal distribution is due to an impairment of retrograde transport of lysosomes in JLP knockdown cells. To test this hypothesis, we performed rescue experiments wherein cells were

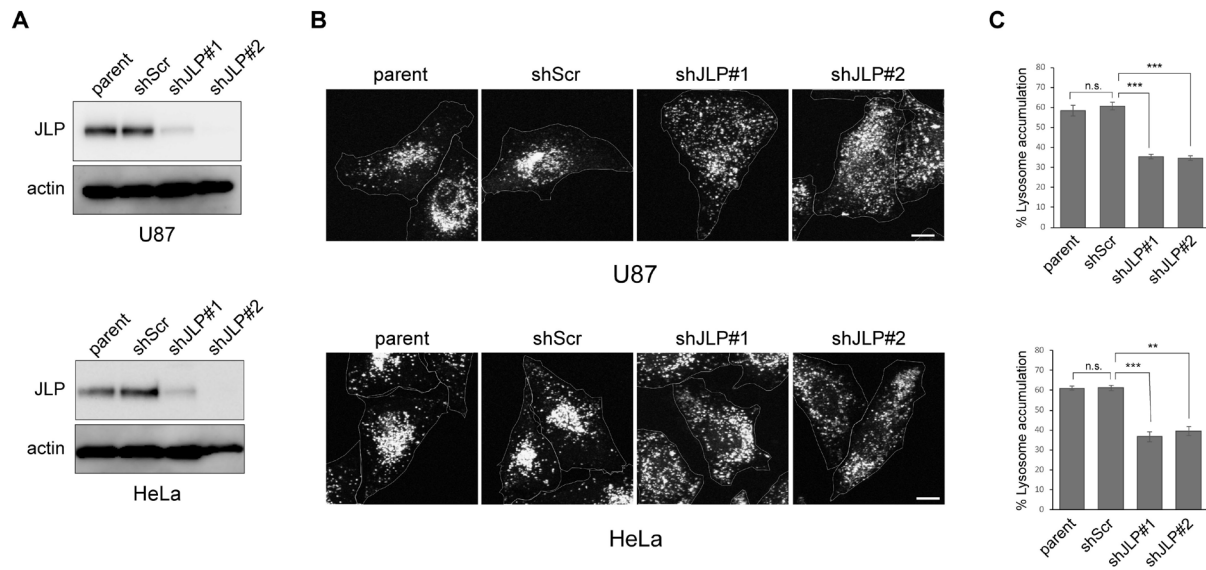


Figure 1. JLP knockdown impairs lysosomal accumulation around the perinuclear region. (A) U87 and HeLa cells were transduced or not (parent cells) with lentiviral vectors for shScr, shJLP#1, or shJLP#2, as indicated, and the expression levels of JLP were analyzed by western blotting with an anti-JLP antibody. Actin was utilized as the loading control. (B) U87 and HeLa cells expressing shScr, shJLP#1, shJLP#2, and their corresponding parent cells, as indicated, were immunostained with an anti-LAMP-1 antibody. Images were obtained by confocal microscopy. Scale bars, 10 μ m. (C) Quantification of the lysosome distribution imaged in (B). Quantitative data are expressed as mean \pm S.E.M. of three independent experiments. The perinuclear region was defined as 0–5 μ m from the nuclear rim. At least 20 cells per experiment condition were analyzed. ** $p < 0.01$; *** $p < 0.001$; n.s., not significant.

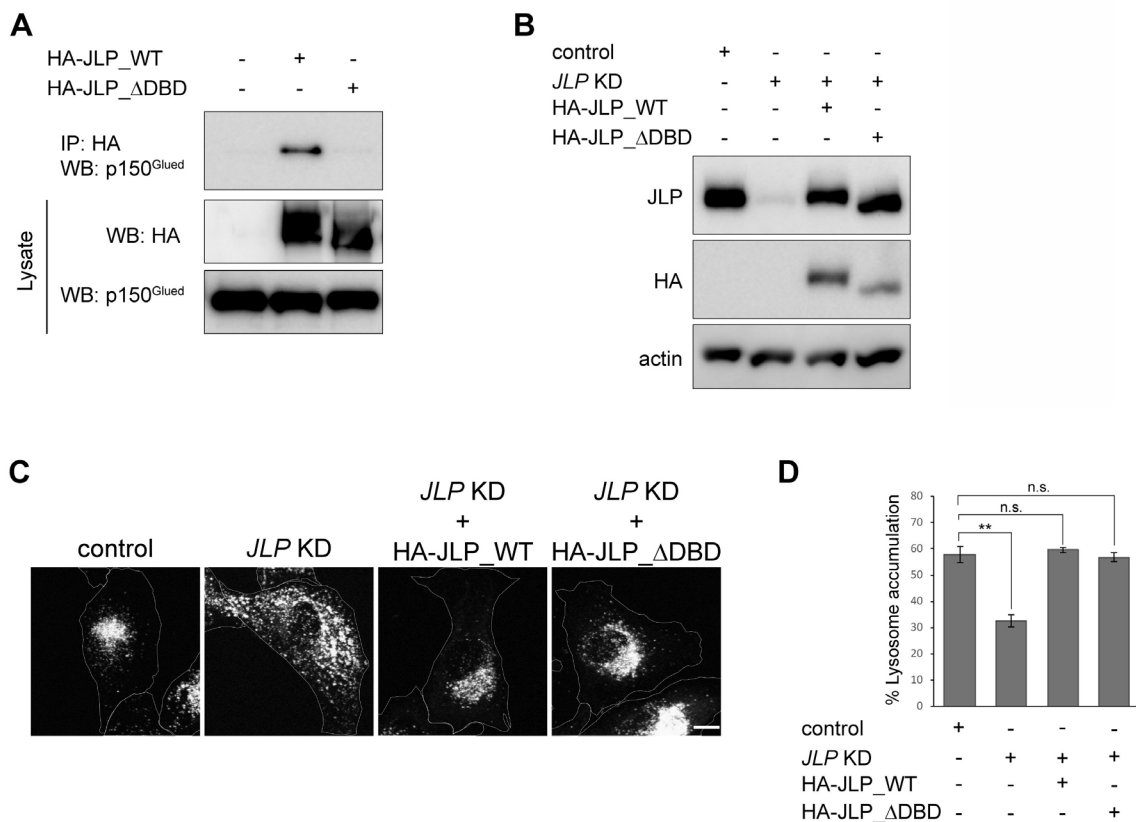


Figure 2. JLP is involved in lysosome localization independently from its interaction with dynactin p150^{Glued}. (A) HA-JLP-WT or HA-JLP- Δ DBD were transiently expressed in HEK293T cells as indicated, immunoprecipitated (IP) with anti-HA antibody beads and subjected to western blotting (WB) using an anti-p150^{Glued} antibody. The expression of HA-JLPs (middle panel) and of p150^{Glued} (lower panel) in total cell lysates is shown. (B) U87 cells expressing either shScr (control) or shJLP#2 (JLP KD), alone or together with HA-JLP_WT or HA-JLP_ Δ DBD as indicated, were analyzed by western blotting with anti-JLP and anti-HA antibodies. Actin was used as a loading control. (C) The U87 cells in (B) were immunostained with an anti-LAMP-1 antibody and observed by confocal microscopy. Scale bar, 10 μ m. (D) The results of lysosome distribution in (C) were quantified as in Figure 1C. ** $p < 0.01$; n.s., not significant.

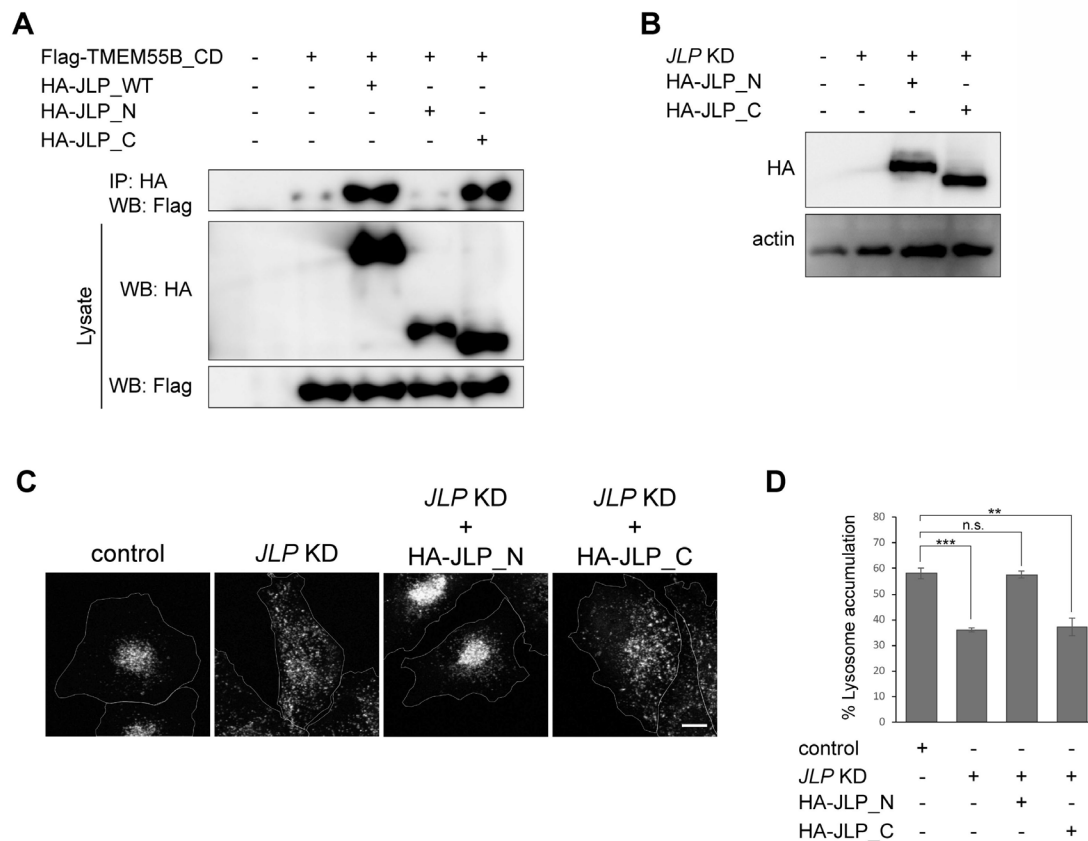


Figure 3. JLP-TMEM55B interaction is dispensable for lysosome localization. (A) Flag-TMEM55B_CD was transiently co-expressed with HA-JLP_WT, HA-JLP_N, or HA-JLP_C as indicated in HEK293T cells, immunoprecipitated (IP) with anti-HA antibody beads, and subjected to western blotting (WB) using an anti-Flag antibody. The expression of HA-JLPs (middle panel) and of Flag-TMEM55B_CD (lower panel) in total cell lysates is shown. HA-JLP_N and HA-JLP_C were expressed using the pCL20c-CMV vector. (B) U87 cells expressing shJLP#2 (*JLP* KD) alone or together with HA-JLP_N or HA-JLP_C as indicated were analyzed by western blotting with an anti-HA antibody. Actin was utilized as a loading control. (C, D) The U87 cells in (B) were immunostained with an anti-LAMP-1 antibody (C), and the results were quantified as in Figure 1C (D). ** $p < 0.01$; *** $p < 0.001$; n.s., not significant. Scale bar, 10 μ m.

transduced with lentiviral particles containing HA-tagged wild-type JLP (HA-JLP_WT) or its mutant lacking DBD (HA-JLP_ΔDBD), both of which are shJLP#2-resistant (Figure 2). We confirmed that HA-JLP_ΔDBD was unable to interact with dynactin p150^{Glued} (Figure 2A). However, as shown in Figure 2C and D, both HA-JLP_WT and HA-JLP_ΔDBD reversed the lysosome distribution in *JLP* knockdown cells. The expression levels of JLP were comparable among the U87 cells expressing shScr, HA-JLP_WT, and HA-JLP_ΔDBD (Figure 2B). In addition, the percent of HA-positive cells were over 90% in cells transduced with lentivirus for HA-JLP_WT or HA-JLP_ΔDBD (Figure S2A, <http://www.ddtjournal.com/action/getSupplementalData.php?ID=53>). Taken together, these results suggest that the role of JLP in lysosome localization is independent from its interaction with dynactin p150^{Glued}.

3.3. JLP mutants lacking the TMEM55B-binding domain, but not the N-terminal region, restore altered lysosome localization in *JLP* knockdown cells

As TMEM55B has been reported to recruit JLP to the

lysosomal surface, inducing retrograde transport of lysosomes (4), we asked whether the JLP-TMEM55B interaction is involved in lysosome localization. As a first step to examine this possibility, we generated expression plasmids for HA-tagged N- and C-terminal half deletion mutants of JLP (named HA-JLP_N and HA-JLP_C, respectively) in addition to HA-tagged full-length wild-type JLP, HA-JLP_WT. In HEK293T cells we transiently co-expressed one of the HA-JLPs together with Flag-tagged TMEM55B and performed immunoprecipitation-western blot analysis. Through this approach, we found that the C-terminal half, but not the N-terminal half, of JLP is responsible for interaction with TMEM55B (Figure 3A). We then performed rescue experiments using these JLP deletion mutants using the same approach detailed above for HA-JLP-ΔDBD. The expression levels of lentivirus-transduced HA-JLP_N and HA-JLP_C were comparable (Figure 3B), and the percent of HA-positive cells were over 90% in cells transduced with the respective lentivirus particles (Figure S2B, <http://www.ddtjournal.com/action/getSupplementalData.php?ID=53>). We observed that HA-JLP_N, but not HA-JLP_C, restored the localization of lysosomes in *JLP*

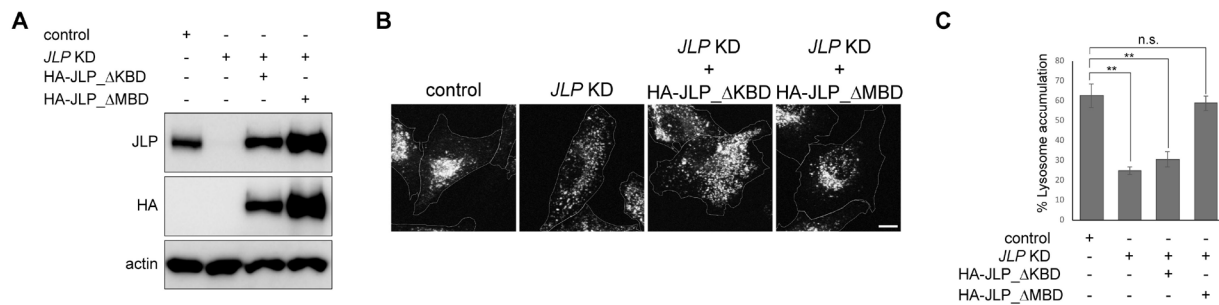


Figure 4. A JLP mutant lacking KBD, but not MBD, fails to reverse the altered lysosomal distribution in *JLP* knockdown cells. (A-C) U87 cells expressing either shScr (control) or shJLP#2 (*JLP* KD), alone or together with HA-JLP_ΔKBD or HA-JLP_ΔMBD as indicated were analyzed by western blotting with an anti-HA antibody (A). The cells in (A) were immunostained with an anti-LAMP-1 antibody (B), and the results were quantified as in Figure 1C (C). Actin was used as a loading control. ** $p < 0.01$; n.s., not significant. Scale bar, 10 μ m.

knockdown cells (Figures 3C and D). Collectively, these results suggest that the JLP-TMEM55B interaction is unrelated to lysosome localization and that the N-terminal half of JLP is necessary and sufficient for the regulation of lysosome localization.

3.4. JLP may play a role in retrograde movement of lysosomes through interaction with KHC

It is known that JLP contains two domains, KBD and MBD, in its N-terminal region (10,14). We, therefore, examined whether KBD and/or MBD are involved in lysosome localization using HA-tagged shJLP#2-resistant JLP mutants lacking KBD (HA-JLP_ΔKBD) or MBD (HA-JLP_ΔMBD). After confirming the expression levels of HA-JLP_ΔKBD and HA-JLP_ΔMBD (Figure 4A), and the transduction efficiency of the lentiviral particles in cells (Figure S2C, <http://www.ddtjournal.com/action/getSupplementalData.php?ID=53>), we carried out rescue experiments. Interestingly, HA-JLP_ΔMBD, but not HA-JLP_ΔKBD, restored the altered lysosomal distribution in *JLP* knockdown cells (Figure 4B and C), suggesting that the JLP-KHC interaction may play a role in the retrograde movement of lysosomes.

3.5. *JLP* knockdown impairs autophagosome-lysosome fusion

Because lysosome localization is important for autophagy (20), we examined whether *JLP* knockdown affects the induction of autophagy. We first assessed autophagy based on the level of LC3-II, which has been reported to closely reflect the number of autophagosomes (21). Control cells expressing shScr were placed under amino acid starvation to induce autophagy, then incubated in the presence or absence of chloroquine, an inhibitor of autophagosome-lysosome fusion. An increased amount of LC3-II was observed in chloroquine-treated control cells, which was further increased by treatment of the cells with both amino acid starvation and chloroquine (Figure 5A). A similar expression profile of LC3-II was observed in amino acid-starved *JLP* knockdown cells (Figure 5A),

suggesting that *JLP* knockdown has little or no effect on LC3-II generation in amino acid starvation-induced autophagy. We next asked whether JLP is involved in autophagosome-lysosome fusion, part of the maturation process of autophagosomes. To examine this possibility, we employed a unique, tandem fluorophore reporter, mRFP-GFP-LC3 (22). We expressed mRFP-GFP-LC3 by lentiviral transduction in the control and *JLP* knockdown cells, performed amino acid starvation, and immunostained cells with an antibody against LAMP-1. Control cells showed a good colocalization of mRFP puncta and LAMP-1 signals, and low colocalization of punctate signals of LAMP-1-positive mRFP with GFP (Figures 5B-5D). By contrast, in *JLP* knockdown cells we observed low colocalization of mRFP puncta and LAMP-1 signals, and good colocalization of punctate signals of LAMP-1-positive mRFP with GFP (Figures 5B-5D). The altered profile observed in the *JLP* knockdown was reversed by the expression of HA-JLP_WT or HA-JLP_N, but not HA-JLP_ΔKBD (Figures 5B-5D). Taken together, these results suggest that JLP plays an important role in autophagy by regulating lysosome localization.

4. Discussion

In the present study, we demonstrated that JLP plays an important role in lysosome localization and autophagy, as evidenced by the lysosome distribution and the impairment of autophagosome-lysosome fusion in *JLP* knockdown cells.

It is well known that lysosomes move toward the cell center using dynein motors (1,3). Additionally, JLP has been reported to interact with p150^{glued}, a key component of the dynactin complex (23). However, expression of a JLP mutant lacking the binding domain of dynactin p150^{glued} in *JLP* knockdown cells restored the altered lysosomal distribution (Figure 2). JLP has been suggested to interact with p50/dynamitin (23), a component of dynactin complex. Therefore, it is possible that other component(s) of the dynein-dynactin complex, including p50/dynamitin, are involved in lysosome

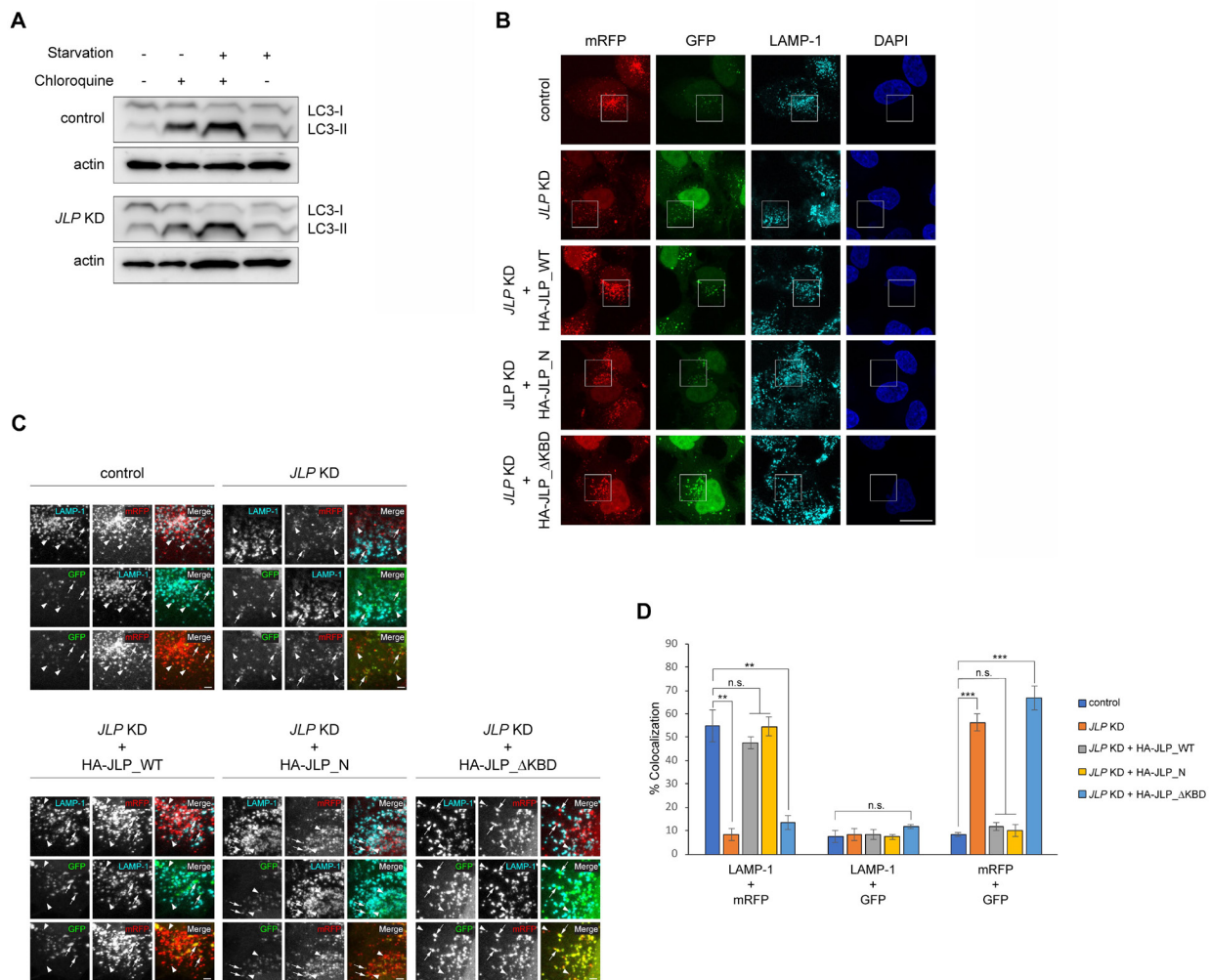


Figure 5. JLP mediates autophagy induction. (A) HeLa cells expressing shScr (control) or shJLP#2 (*JLP* KD) were amino acid starved for 4 hours in the absence or presence of chloroquine, as indicated, and subjected to western blotting with an anti-LC3 antibody. Actin was utilized as a loading control. (B) The control and *JLP* knockdown HeLa cells treated as in (A) were transduced with a lentivirus expressing the mRFP-GFP-LC3 fusion protein, amino acid starved for 4 hours, immunostained with an anti-LAMP-1 antibody, fixed, and analyzed by confocal microscopy. For rescue experiments, *JLP* knockdown HeLa cells were transduced with lentiviruses expressing HA-JLP_WT, HA-JLP_N, or HA-JLP_ΔKBD as indicated together with mRFP-GFP-LC3, amino acid starved for 4 hours, immunostained, fixed, and analyzed as described above. Nuclei were stained with DAPI. Areas in the boxes indicated by dotted-lines are shown at a higher magnification in (C). Scale bar, 20 μ m. (C) Arrowheads indicate typical examples of colocalization signals of LAMP-1 and mRFP. Arrows indicate typical examples of colocalization signals of mRFP and GFP. Scale bars, 2 μ m. (D) The results of the colocalization analyses in (B) and (C) were quantified. At least 20 cells per experimental condition were analyzed. ** $p < 0.01$; *** $p < 0.001$; n.s., not significant.

localization through interaction with JLP.

Willett *et al.* (4) identified the dynein adaptor JLP as a binding protein of the lysosomal transmembrane protein TMEM55B, and proposed that TMEM55B recruits JLP to the lysosomal surface and facilitates dynein-dependent transport of lysosomes under stress conditions, including starvation. In addition, they demonstrated a scattered distribution of lysosomes in cells depleted of TMEM55B or JLP (4). Here we found the

N-terminal half fragment of JLP is necessary and sufficient to rescue the dispersed distribution of lysosomes in *JLP* knockdown cells, although this N-terminal fragment is unable to bind with TMEM55B (Figure 3). It is therefore unlikely that the JLP-TMEM55B interaction is involved in the regulation of lysosome localization. However, we cannot rule out

the possibility that the JLP N-terminal region indirectly interacts with TMEM55B through unknown factor(s). Further studies are needed to clarify the mechanisms of JLP- and/or TMEM55B-mediated lysosome distribution.

We found in this study that a JLP mutant lacking the KBD was unable to reverse the altered distribution of lysosomes in *JLP* knockdown cells (Figure 4). This result might suggest that JLP mediates retrograde transport of lysosomes *via* interaction with kinesin-1. In support of this possibility, Arimoto *et al.* (24) reported that kinesin-1 and UNC-16, an ortholog of mammalian JLP and its family member JSAP1, are required for retrograde transport of various axonal proteins in *Caenorhabditis elegans*. It is also possible that the KBD may have another function, unrelated to kinesin-1, which is necessary for retrograde transport of lysosomes.

This study provides new insight into the regulatory mechanisms of lysosomal trafficking. Our findings contribute to the understanding of how lysosomes exert their multiple functions, potentially enabling the identification of molecular targets for diseases caused by lysosomal dysfunction.

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