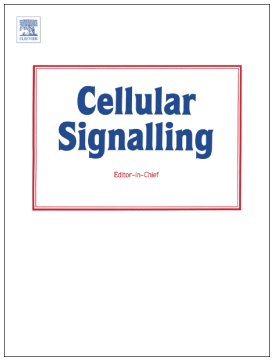

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Autophagy promotes osteoclast podosome disassembly and cell motility through the interaction of kindlin3 with LC3

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Abstract
Osteoclasts are responsible for bone resorption and play an important role in physiological and pathological bone metabolism. Osteoclast migration across bone surfaces is essential for bone resorption, and a previous study demonstrated the role of autophagy in osteoclastogenesis and acid secretion. However, the role of autophagy in osteoclast migration remains unclear. Osteoclast migration requires the successive and rapid assembly and disassembly of podosome rings. In this study, we show that kindlin3, an important adaptor protein in the podosome, can interact with LC3B and undergo autophagy-mediated protein degradation to promote the disassembly of the podosome.
Moreover, further analyses showed that the inhibition of autophagy increased kindlin3 levels and enhanced the interaction between kindlin3 and integrin β3. The over activation of integrins inhibits the disassembly of obsolete podosome rings, resulting in disorganization of the actin cytoskeleton and impaired migration in osteoclasts. Our results show that LC3B affects osteoclast migration and FAK/AKT activation by modulating integrin activation via a kindlin3-mediated inside-out signal from the extracellular matrix. Based on these results, we propose that LC3 is an important target for regulating osteoclast migration.

Key words: LC3B; osteoclast; migration; kindlin3; autophagy

Introduction

Autophagy has pleiotropic functions and has been shown to be involved in multiple important activated of osteoclasts, such as osteoclastic differentiation and osteoclastic-mediated bone resorption [1]. However, it remains unclear whether autophagy participates in the migration of osteoclasts and whether it is responsible for maintaining the balance of the skeletal system.

Osteoclasts (OCs) are a crucial part of the skeletal system and are involved in bone reconstitution and the maintenance of tissue homeostasis. The ability of osteoclasts to migrate and to resorb is based on their adaptable morphology and the formation and degradation of podosomes [2-4]. Podosomes are actin-rich cellular structures that are organized by two distinct parts: a central actin core and a protein cloud that surrounds the core, including actin regulatory proteins such as integrin-associated proteins (vinculin and kindlin3) and kinases (PI3K and Rho GTPases) [5]. Previous studies have shown that in mature osteoclasts, the

podosome evolves into circular patterns called rings, which apply traction forces to the matrix to drive osteoclast migration [6].

Kindlin3 is a member of the kindlin family of focal adhesion proteins encoded by Fermt3. Current studies suggest that the kindlin3 protein is expressed in only the hematopoietic system and activates integrin inside-out signaling by directly binding to the cytoplasmic tails of integrins [7]. Deficiency of kindlin3 (kindlin3-/- mouse model) interferes with integrin activation, which in turn leads to severe bleeding, erythrocyte abnormalities and osteopetrosis [8, 9].

Autophagy is an evolutionarily conserved catabolic process that eliminates proteins, clears senescent organelles, and removes pathogens. Autophagy maintains cell homeostasis and adjusts to nutrient stress and starvation (under stressful conditions) by reusing obsolete cellular components [10]. Microtubule-associated protein LC3 can dispose of superfluous or potentially dangerous cytosolic entities by fusing with lysosomes, which is essential for autophagy. LC3 recognizes a variety of cargos via surface receptors and is involved in cargo selection [11]. There are three subfamilies of LC3 proteins in mammals and we mainly studied the role of the major subtype LC3B in osteoclasts.

In this study, we showed that in osteoclasts differentiated from RAW264.7 cells, LC3B is involved in integrin turnover and affects podosome disassembly via the degradation of kindlin3. We show that LC3B-deficient osteoclasts fail to degrade obsolete podosomes, which in turn leads to an increase in podosome rings and intracellular actin structural disorder, as well as a failure to migrate and degrade the extracellular matrix. These findings suggest that LC3B is necessary for podosome turnover and osteoclast migration.

Results

Inhibition of autophagy leads to severe osteoclast dysfunction

By observing and measuring the migration trench of osteoclasts on dentin slices, we showed that the distance osteoclasts migrated on these slices decreased after osteoclast autophagy was inhibited by Lys05 treatment (figure 1A). The migration of osteoclasts is dependent on podosomes and podosome rings. To explore the role of autophagy in osteoclast migration, we examined the changes in podosome rings in osteoclasts after autophagy inhibition. We observed that osteoclasts displayed multiple actin ring structures and that the fluorescence intensity of actin increased in the autophagy inhibition group (figure 1B). Subsequently, we performed the same experiment with primary osteoclasts from mice and showed that the actin structure was also disordered and that there were multiple ring structures (Supplemental Fig. 1A). We examined the inhibitory effect of three different LC3B small interfering RNAs (siRNAs). After 48 h of siRNA transfection in osteoclasts, the inhibition rates of si-2 and si-3 reached 75% (figure 1C), and these siRNAs were used in subsequent experiments. After inhibiting osteoclast autophagy with LC3B siRNAs, we observed that the fluorescence intensity of actin and the width of the osteoclast podosome ring was increased. Furthermore, the number of podosome rings in osteoclasts was increased, and the structure had become chaotic (figure 1D-H). The number of rings in the silc3-treated cells increased, and the actin rings were dispersed in the cells. Atg5 is an important protein that acts upstream of autophagy.

We also used small interfering RNAs to reduce the expression of ATG5 and observed that the width of podosome rings increased and that the fluorescence intensity of actin increased, similar to the LC3 interference results (Supplemental Fig. 1B, C, D, E).

Kindlin3 accumulates in osteoclasts after autophagy inhibition

After using Lys05 or LC3B siRNA to inhibit autophagy, we observed that kindlin3, an important adaptor protein in the podosome, exhibited significant accumulation in osteoclasts (figure 2A, B). Through immunofluorescence assays, we also observed that when the autophagy of osteoclasts was inhibited by LC3B siRNA, the levels of kindlin3 in osteoclasts increased significantly and was accompanied by an increase in podosome rings (figure 2C). To assess the stability of kindlin3, we simultaneously stimulated autophagy by starvation and inhibited autophagy by Lys05 treatment. In the experimental group treated with an autophagy inhibitor, we found that kindlin3 accumulated during the first hour and degraded slowly. Because autophagy was not inhibited in the control group, smooth and rapid kindlin3 degradation was observed in these cells. The trend of kindlin3 degradation was consistent with that of the protein p62, which is encoded by the gene sequestosome 1 (SQSTM1) and is continually degraded during autophagy, leading us to hypothesize that changes in the level of autophagy may affect the stability of kindlin3 (figure 2D, E). We inhibited the expression of kindlin3 and found no changes in the level of LC3 in osteoclasts (figure 2F). These findings indicate that autophagy can regulate kindlin3 stability and function in cells by affecting its degradation.

Kindlin3 interacts with LC3B and undergoes autophagy-mediated protein degradation in osteoclasts

LC3B plays a role in the elongation process and binds autophagic substrates during autophagosome formation [12, 13]. In subsequent experiments, we investigated whether LC3B plays a direct role in the degradation of kindlin3. Through immunofluorescence staining analyses, we observed that kindlin3 protein was clearly localized to osteoclast autophagosomes. Compared with the control group, when Lys05 was used to inhibit osteoclast autophagy, significant LC3B and kindlin3 accumulation was observed, and their colocalization increased significantly (figure 3A). LAMP-2 is used as marker for autophagolysosomes. We observed that when Lys05 was used to inhibit autophagy, the levels of kindlin3 and LAMP-2 around the nuclei of osteoclasts significantly increased, and these proteins exhibited notable colocalization (Supplemental figure. 2A). We performed a coimmunoprecipitation (CO-IP) assay to confirm the presence of a complex formed by kindlin3 and LC3 in osteoclasts. We successfully pulled down LC3B with kindlin3 (figure 3B) and vice versa (figure 3C). In addition, we also performed CO-IP experiments in 293T cells by transfecting cells with constructs to express His-kindlin3 and Myc-LC3B. As shown in figure 3D, Myc-LC3B could pulldown His-kindlin3 and vice versa (figure 3E) in 293T cells. These data demonstrate that LC3 can recognize and bind to kindlin3 and transfer it to the autophagosome for degradation.

Autophagy inhibition activates the kindlin3-integrin pathway in osteoclasts

Integrin αvβ3 is widely distributed and expressed on the surface of a variety of mesenchymal

and blood vascular cells, such as macrophages, osteoclasts and endothelial cells [14]. Through CO-IP assays, we showed that inhibiting LC3B protein expression facilitated the binding of kindlin3 to integrin β3 (figure 4A). The same results were also observed in immunofluorescence assays with osteoclasts. When osteoclast autophagy was inhibited, the colocalization of kindlin3 and integrin beta3 increased significantly (figure 4B). Next, we analyzed whether the accumulation of kindlin3 protein due to knockdown of LC3B affects integrin-mediated adhesion signaling. Rapid activation of focal adhesion kinase (FAK) and the tyrosine kinase SRC are often hallmark signaling events associated with ligand binding to integrins [15]. We treated osteoclasts with two different LC3B siRNAs and observed that the phosphorylation levels of FAK (Y397) and SRC (Y416) were significantly increased (figure 4C). We also assessed the status of downstream signaling proteins, such as AKT and ERK. As shown in figure 4D, although no significant changes in ERK signaling pathway proteins were observed, the phosphorylation level of AKT increased significantly. These results suggest that inhibition of LC3 may result in the over activation of integrin and its downstream signaling pathway, potentially explaining why the podosome ring adheres tightly to the extracellular matrix and is not easily disassembled.

LC3B-mediated autophagy inhibits osteoclast migration via kindlin3

Does LC3B regulate the activation of integrin αvβ3 through kindlin3? Recovery experiments were performed to verify the essential role of LC3B in kindlin3-mediated integrin activation. After knockdown of kindlin3 levels in osteoclasts, the phosphorylation levels of FAK, SRC and AKT decreased. Further analysis showed that when LC3B and kindlin3 were simultaneously knocked down, the decrease in FAK, SRC and AKT phosphorylation resulting from kindlin3 knockdown was abrogated (figure 5A). We also showed that Lys05 can promote the complete recovery of the decrease in FAK, SRC and AKT phosphorylation levels caused by kindlin3 knockdown. Simultaneously, due to the strong effect of Lys05, the level of FAK, SRC and AKT phosphorylation in the recovery group was even higher than that observed in the NC group (Supplemental Fig. 3A). In osteoclasts, immunofluorescence assay results confirmed that the actin cytoskeleton disorder was alleviated by simultaneously inhibiting the expression of LC3B and kindlin3, whereas the width of the rings did not change significantly (figure 5B, C). Osteoclasts were cultured on calcium phosphate-coated plates to measure the distance they migrated. The trench length was measured at 48 and 72 h, and the difference was reported as the 24-h migration distance of osteoclasts. The results suggest that the distance osteoclasts migrated was partially restored by the simultaneous inhibition of LC3B and kindlin3 (figure 5D, E). Subsequently, we cultured osteoclasts on dentin slices and observed that the osteoclasts with LC3 knockdown hardly moved. In contrast, osteoclasts with LC3 and kindlin3 knockdown exhibited partial recovery of their migration activity, but the lengths of the trenches on dentin slices were not as long as those observed for the NC group (figure 5F).

Summary

Autophagy plays an important role in the regulation of osteoclast differentiation and bone

resorption. In the regulation of cell differentiation, autophagy can regulate osteoclastogenesis by Beclin1 and mTOR signaling [1, 16]. In bone resorption, lipidated LC3 assists lysosome localization to the ruffled border, a special plasmalemma construct, to secret lysosomal enzymes and HCl into the extracellular resorptive space [17].

In this study, we show that the autophagy-associated protein LC3B functions in the disassembly of the podosome and the migration of osteoclasts by regulating kindlin3, a significant adaptor protein. In detail, the inhibition of LC3B via siRNA enhanced the interaction between integrin β3 and kindlin3, and the accumulation of kindlin3 may be an important cause of excessive integrin β3 activation. Osteoclasts with increased kindlin3 levels showed increased numbers of podosome rings, increased ring thickness and a disordered actin structure, which resulted in impaired migration and bone resorption. Thus, LC3 not only promotes enzyme secretion for bone matrix resorption but also promotes osteoclast migration in a manner dependent on kindlin3 and integrin αvβ3.

Lys05 is a novel type of dimeric chloroquine autophagy inhibitor that can inhibit autophagy by deacidifying the lysosome [18,19]. The effects of Lys05 treatment and LC3 knockdown on osteoclasts were similar. First, in both the Lys05 treatment and LC3 knockdown groups, the migration of osteoclasts decreased, the actin structure in the cells significantly increased and the actin structural morphology was disordered. Second, after inhibition of autophagy, the colocalization of actin and its migration-associated proteins in podosome rings, such as vinculin and kindlin3, was no longer obvious. However, we observed difference in the effect that Lys05 treatment and LC3 knockdown had on the migration of osteoclasts. After Lys05 treatment of osteoclasts, multiple irregular actin rings formed from the inner to the outer regions of the cytoplasm, possibly due to the old actin rings being unable to be degraded in time. When LC3 knocked down, multiple actin rings were scattered in osteoclasts, the number and widths of which were much greater than those of normal osteoclasts. We preliminarily speculate that Lys05 is a broad spectrum autophagy inhibitor. After Lys05 treatment, the degradation of many proteins in osteoclasts was simultaneously inhibited, resulting in severe physiological and functional changes in cells. SiLC3B inhibits LC3B protein expression, and the change in cell migration in the LC3B knockdown cells was not as dramatic as that caused by the Lys05 treatment.

However, LC3-mediated degradation of kindlin3 is not the only mechanism by which autophagy regulates cell migration. Previous studies have demonstrated that in highly metastatic tumor cells, such as 4T1 mouse mammary tumors, LC3 can promote focal adhesion disassembly by degrading paxillin and inducing cell spreading, migration, and invasion. However, when autophagy is inhibited, no changes in integrin signaling pathways, especially FAK phosphorylation levels, are observed [20]. M. Cecilia Caino observed that under nutrient deprivation conditions, mitochondrial HSP90 preserves cytoskeletal dynamics by reinforcing the phosphorylation of FAK and preventing autophagy [21]. This difference may be due to differences in cellular systems and migration patterns. Since autophagy can affect cell migration through different signaling pathways, the migration function of osteoclasts was not completely restored by the inhibition of both LC3B and kindlin3. Our

results show that LC3B play a regulatory role in the process of osteoclast migration at least partly through the kindlin3-integrin signaling pathway.

Ruizhi He showed that in pancreatic cancers, TGF-β-mediated autophagy can promote the endocytosis of Itgα5β1, the disassembly of focal adhesions and accelerate the loss of P-FAK [22]. Nevertheless, in our mass spectrometry results (not shown here), we did not detect the binding of LC3B protein to integrin-related proteins, especially β3. Therefore, we preliminarily hypothesize that in osteoclasts, LC3B regulates integrin activation through adaptor molecules rather than through a direct interaction with the integrin.

Podosome ring-driven migration is essential to osteoclast-mediated bone resorption [23]. Osteoclast migration is more complicated than the migration of other cells with podosome structures, such as dendritic cells (DCs) [22, 24], because it relies on the ring structure of podosomes instead of individual podosomes. When an osteoclast migrates, the ring opposite to the migration direction becomes vulnerable and eventually degrades [6]. Although previous studies have shown that podosomes are dispensable for osteoclast differentiation and migration on polyacrylamide gels [25], we speculate that the composition of polyacrylamide gels is not common in vivo, and the migration mechanism of osteoclasts on gel may be different from that on bone.

Podosome rings dissolve, and αvβ3 moves to the leading edge of the lamellipodium [26]. Although mature osteoclasts express integrins 1 and 2, previous data suggest that αvβ3 integrins are the major adhesion proteins and mediate substrate attachment in osteoclasts [27]. Kindlin3 alters the conformation of αvβ3 to its high-affinity conformation by directly interacting with the second NPxY motif of integrin β tails, and it is required for αvβ3 integrin to modify cell contractility [28]. Unlike the expression of other members of the widely expressed kindlin family, kindlin3 expression is limited to hematopoietic cells, including osteoclasts, which differentiate from hematopoietic progenitors of the myeloid lineage. The dynamic balance of kindlin3 in osteoclasts is very important. The adaptor function of kindlin3 is required for osteoclast-mediated bone resorption [27, 29]. Osteopetrosis has been observed in both patients and kindlin3-deficient mice [8, 9]. Significant colocalization of kindlin3 with podosome rings was observed in our study. In nonhematopoietic cells, kindlin1 and kindlin2 can also colocalize with focal adhesions [30, 31], indicating that the function of kindlin family proteins is highly conserved.

The interaction between LC3B and kindlin3 is a novel molecular mechanism that is implicated in integrin activation, a crucial process that is necessary for actin reconstruction, osteoclast migration and bone resorption. Furthermore, little is known about kindlin3, and determining these mechanisms will be of crucial importance for understanding the role of kindlin3 in podosome biology and its contribution to the pathophysiology of osteoclast disorders, such as osteopetrosis and LAD-III syndrome. Although it is necessary to further study the adaptive role of autophagy in bone metabolism, our findings suggest an innovative strategy for the development of novel therapeutic agents against skeletal abnormalities.

Acknowledgments
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Highlights
1. A novel interaction between LC3B and kindlin3 in osteoclasts was discovered.
2. LC3B can regulate the activation of integrin β3 through kindlin3 in osteoclasts.
3. LC3B can affect osteoclast migration by regulating podosome degradation and may represent a new treatment for bone-related diseases caused by the dysfunction of osteoclasts.

Methods
Reagents and antibodies
Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Lys05 was purchased from Selleck (Houston, TX, USA). Recombinant RANKL was purchased from R&D Systems (Minneapolis, MN, USA). A Tartrate-Resistant Acid Phosphatase kit was purchased from Sigma (MO, USA). Antibodies against AKT, phospho-AKT (Ser473), FAK, phospho-FAK (Tyr397), KINDLIN3, vinculin, SRC, phospho-SRC family (Tyr416), p62, and LC3B were purchased from CST (Danvers, MA, USA). RAW264.7 cells were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China).

Mice
Eight-week-old C57Bl/6J mice were purchased from the Shandong Laboratory Animal Center and were raised under specific pathogen-free conditions. All procedures and experiment were approved by the University Committee for Animal Resources.

Cell culture and migration assay
Mouse macrophage RAW264.7 cells were cultured in DMEM supplemented with 10% FBS and RANKL (50 ng/mL) for osteoclastogenesis. Osteoclast migration was measured as described previously (22). Briefly, osteoclasts were placed on calcium phosphate-coated plates supplemented with RANKL (50 ng/mL). The length of each trench was measured using ImageJ at 48 and 72 h, and the difference between the two measurements was reported as the distance osteoclasts migrated in 24 h.

Osteoclast differentiation
Bone marrow cells were isolated from the bone marrow of 8-week-old C57B mice as described previously [32,33]. Briefly, the cells were cultured in α-MEM supplemented with 10% heat-inactivated fetal calf serum and 30 ng/mL M-CSF for 72 h. After removing the non-adherent cells, the adherent cells were cultured for 10 days in α-MEM containing 30 ng/mL MCSF and 50 ng/mL RANKL, where the culture medium was changed every two days.

Osteoclast pit formation assay

The osteoclasts differentiated from RAW264.7 cells were spread onto dentin slices and cultured in DMEM supplemented with 10% FBS and RANKL (50 ng/mL). After the osteoclasts were attached to the dentin slices, Lys05 was added to the culture medium of the experimental group. The cells on the slices were removed with a 5% sodium hypochlorite solution, after which the slices were washed twice with water and stained with toluidine blue for 5 min. Reflected light microscopy was used to image the trenches in surfaces of each dentin slice, and the lengths of the trenches were measured with ImageJ[34].

Western blot analysis

Osteoclasts were lysed using RIPA Lysis Buffer for 50 min on a shaker. The proteins were then separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Non-specific binding of the antibodies to proteins on the PVDF membrane was blocked with 5% BSA dissolved in TBST, after which the membrane was washed and incubated with each primary antibody overnight at 4°C. Then, the membrane was reacted with HRP-labeled Goat Anti-Rabbit IgG(H+L) for 1 h at 37°C and detected with a FUSION SOLO S SYSTEM. The total protein samples were used for analyses of kindlin3, LC3, GAPDH, Src, p-SRC, FAK, p-FAK, ERK, p-ERK, p62, AKT and p-AKT.

RNA isolation and real-time PCR

Total RNA was extracted from osteoclasts using TRIzol reagent. A BeyoRT™ III First Strand cDNA Synthesis Kit was used to obtain cDNA, BeyoFast™ SYBR Green qPCR Mix was for PCR amplification and a RocheLightCycler480 system was used to detect the differential expression of genes.

Immunofluorescence staining

After fixation, osteoclasts were permeabilized in 0.5% Triton X-100 and incubated with 1% bovine serum albumin. Then, the cells were incubated overnight with primary antibodies against kindlin3, vinculin, actin or LC3B. After washing with PBS, the cells were labeled with an Alexa Fluor 488- or Alexa Fluor 647-conjugated antibody. Cell nuclei were stained with DAPI, and images were obtained with an Olympus Fluoview FV3000 and analyzed using cellSens Dimension.

Plasmid and small interfering RNA (siRNA) transfection

Osteoclasts differentiated from RAW264.7 cells were transfected with siRNAs specific to LC3B, ATG5, kindlin3 or with nonspecific siRNAs as a negative control for 48 h using HiPerFect reagent.

His-Kindlin3 and Myc-LC3B plasmids were transfected into 293T cells for 48 h using Lipofectamine 2000.

Coimmunoprecipitation assays (CO-IP)

CO-IP assays were performed as described previously (35). Cell lysates were incubated with protein A/G beads to eliminate nonspecific binding. Subsequently, cell lysates were subjected

to immunoprecipitation with kindlin3 (1:100), LC3B (1:100), His-tag (1:50), and Myc-tag (1:200) antibodies followed by incubation with protein A/G beads. The beads were then rinsed by IP Lysis/Wash Buffer for two times and boiled to isolate proteins. Finally, the proteins were analyzed by western blot.

Western blot data were analyzed by GraphPad Prism Version 8.0 (USA). All the normally distributed results are given as the mean±S.D. Western blot, immunofluorescence staining, and migration assays were repeated at least 3 times with similar results. $P < 0.05$ was considered statistically significant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

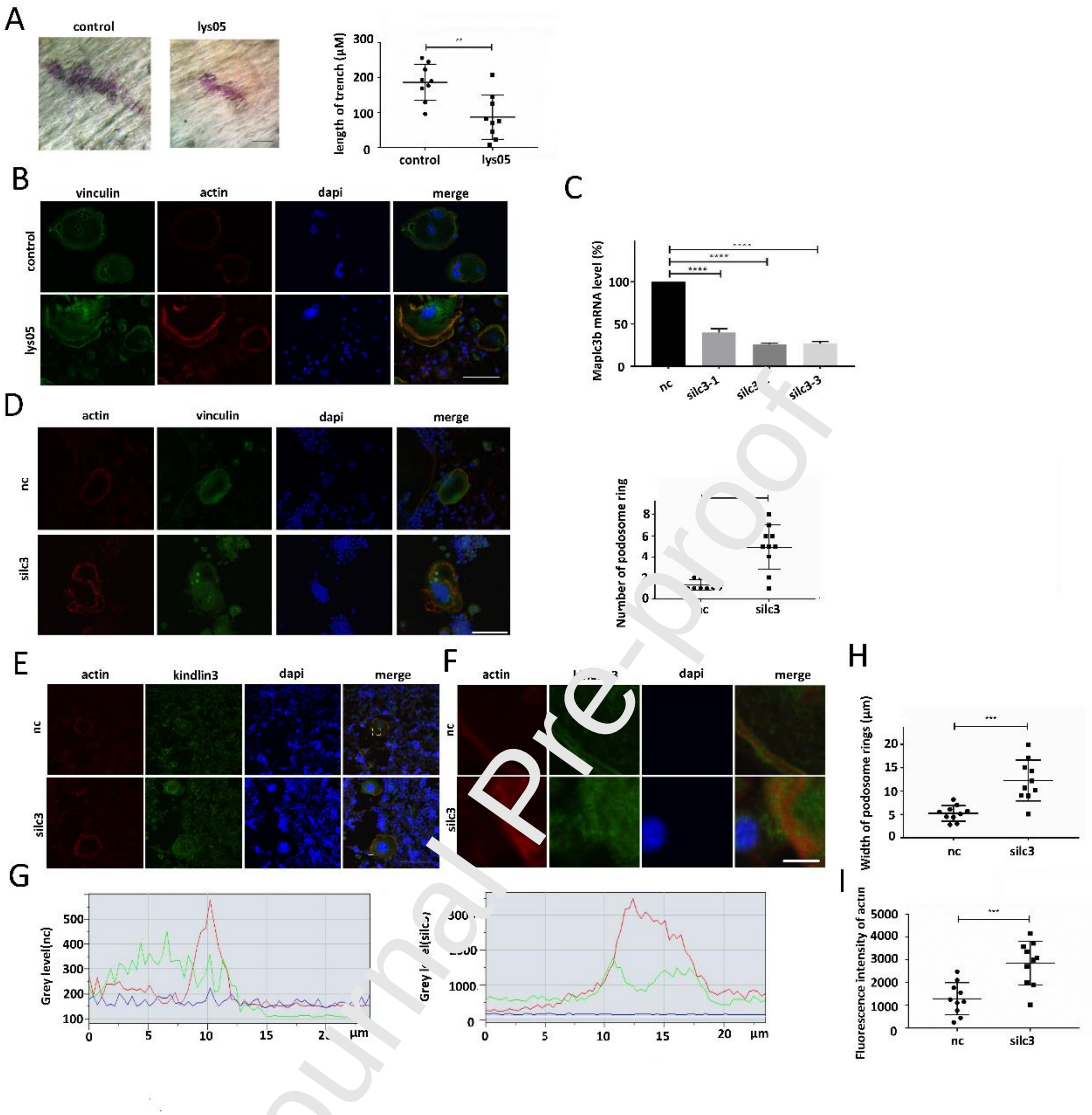


Figure 1 Autophagy inhibition leads to severe osteoclast dysfunction

(a) Raw264.7 cells were incubated with RANKL (50 ng/ml) on slices for 4 days. Osteoclast autophagy was inhibited by 2μM Lys05 treatment for 72 h, and the migration distance of osteoclasts on the dentin was stained by toluidine blue and measured by ImageJ; marker bars, 100 μm. Data are presented as means ± SD. n = 10. **P<0.01

(b) Osteoclast autophagy was inhibited by 2μM Lys05 treatment for 48 h; podosome rings are stained for actin (red) and vinculin (green); the nuclei were stained with DAPI; marker bars, 100 μm. The results shown are representative data of three independent experiments.

(c) Three different kinds of LC3B siRNA were transferred into osteoclasts, respectively. After 48 h, the total RNA was extracted from lysed cells, and the expression of LC3B was measured by quantitative RT-PCR. Data are presented as means ± SD. ****P<0.0001

(d) Osteoclast was transfected with LC3B siRNA intermixture (siLC3B-2 and siLC3B-3) or negative control; podosome rings are stained for actin (red) and vinculin (green); the nuclei

were stained with DAPI; marker bars, 100 μm. The results shown are representative data of three independent experiments. Podosome rings in each osteoclast were counted. Data are presented as mean ± SD. n = 10. ****P <0.0001.

(e,f) LC3B siRNA intermixture (siLC3B-2 and siLC3B-3) and negative control were transfected into osteoclasts using siRNA Transfection Reagent; podosome rings are stained for actin (red) and vinculin (green); the nuclei were stained with DAPI. The marker bars for (f) and (g) are 100um and 10um, respectively. The results shown are representative data of three independent experiments.

(g-i) The fluorescence intensity of actin and the width of podosome rings were measured by cellSens Dimension. Data are presented as mean ± SD. n = 10. ***P<0.001.

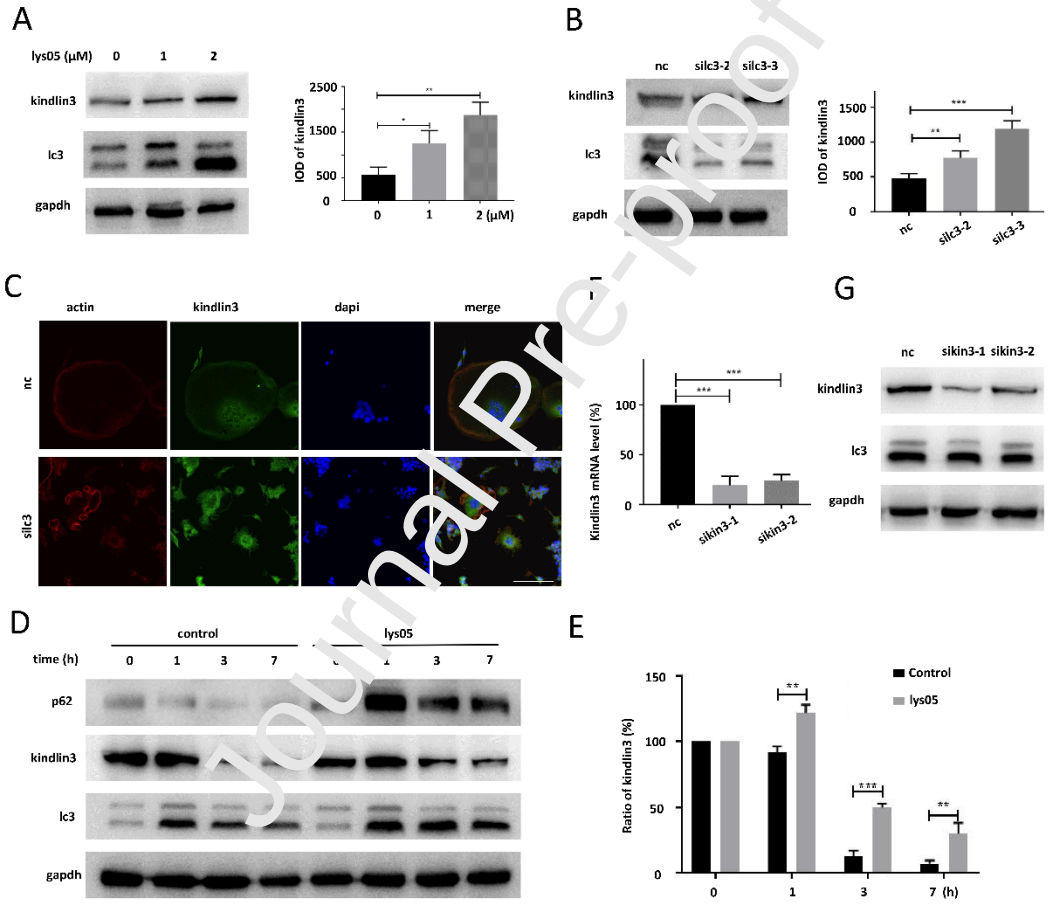


Figure 2 Autophagy inhibition leads to kindlin3 accumulation

(a-b) RAW264.7 cells were seeded in 6-well plates and treated with RANKL (50 ng/mL) for 5 days. Inhibition of autophagy via LC3B siRNA or pharmacological inhibitor Lys05 treatment in osteoclasts. Western blot analysis of kindlin3 in the control and autophagy-inhibited groups.

IOD of kindlin3 was analyzed by ImageJ. Data are presented as means ± SD of three independent experiments; *P < 0.05, **P < 0.01, ***P<0.0001.

(c) Osteoclast autophagy was transfected with LC3B siRNA intermixture (siLC3B-2 and siLC3B-3) and negative control for 48 h. Immunostaining analysis of actin (red) structures and kindlin3 (green) expression in osteoclasts; marker bars, 100 μm. The results shown are representative data of three independent experiments.

(d) Osteoclast autophagy was activated by culture in serum-free medium; Lys05 was added to the autophagy inhibition group. Cells were harvested at each time point, and p62, LC3B and kindlin3 proteins were detected by western blot. The densitometry analysis of kindlin3 is presented in the graph (e) with the 0 h point as the loading control. Data are presented as means ± SD of three independent experiments; **P < 0.01, ***P < 0.001.

(f) Kindlin3 siRNA-1, siRNA-2 and negative control were transferred into osteoclasts. After 48 h, the total RNA was extracted from lysed cells, and the expression of kindlin3 was measured by quantitative RT-PCR. Data are presented as means ± SD. ***P<0.001

(g) Kindlin3 siRNA and negative control were transfected into osteoclasts using siRNA Transfection Reagent. The changes in LC3B protein levels were analyzed by western blot. The results shown are representative data of three independent experiments.

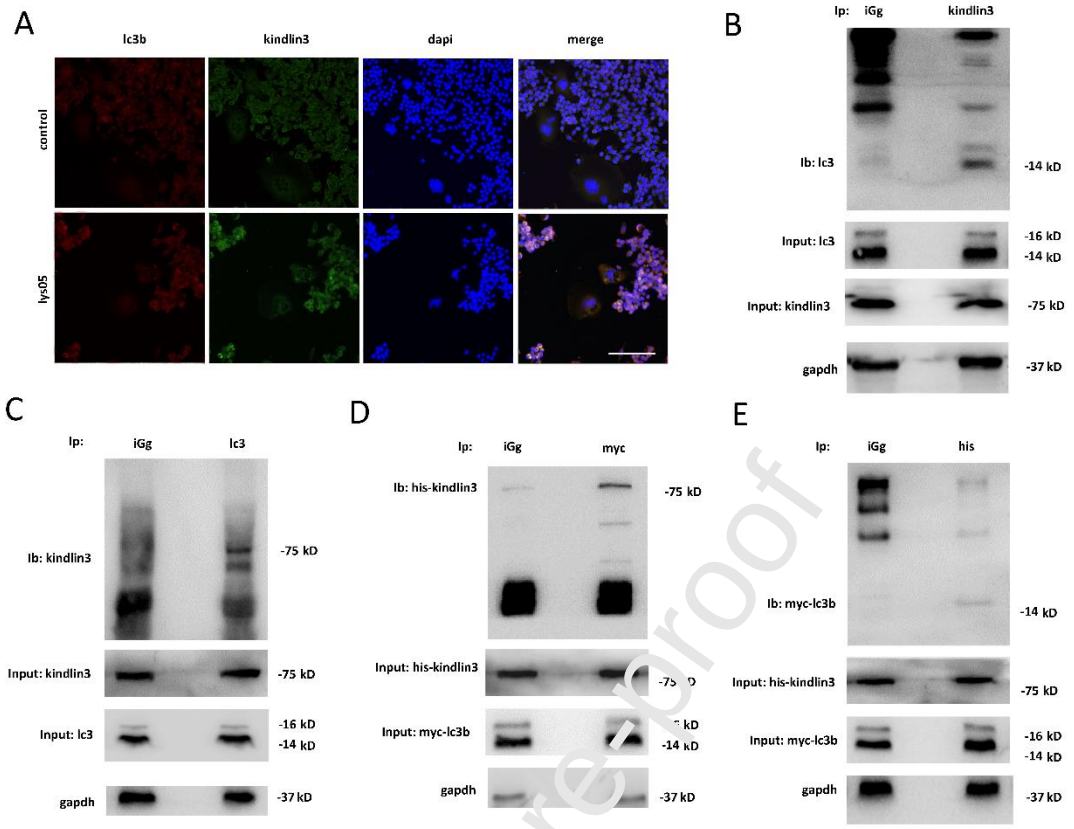


Figure 3 LC3B interacts with kindlin3 in osteoclasts

(a) Osteoclasts were incubated with or without Lys05 in the presence of RANKL (50 ng/ml). Co-immunostaining analysis of kindlin3 (green) and LC3B (red) distribution in mature osteoclasts; the nuclei were stained with DAPI; marker bars, 100 μm.

(b, c) Osteoclasts were subjected to immunoprecipitation (IP) using a kindlin3 antibody (1:100)or LC3B antibody (1:100). As controls, proteins were precipitated with the control IgG derived from the same species.

(d, e) 293T cells were transfected with His-kindlin3 and Myc-LC3B. After 48 h of transfection, the cells were immunoprecipitated (IP) with a His (1:50) and a Myc (1:200) antibody, respectively. Rabbit IgG was used as a negative control. Immunoblots (IB) with rabbit anti-Myc and rabbit anti-His.

The above results are representative data of three independent experiments.

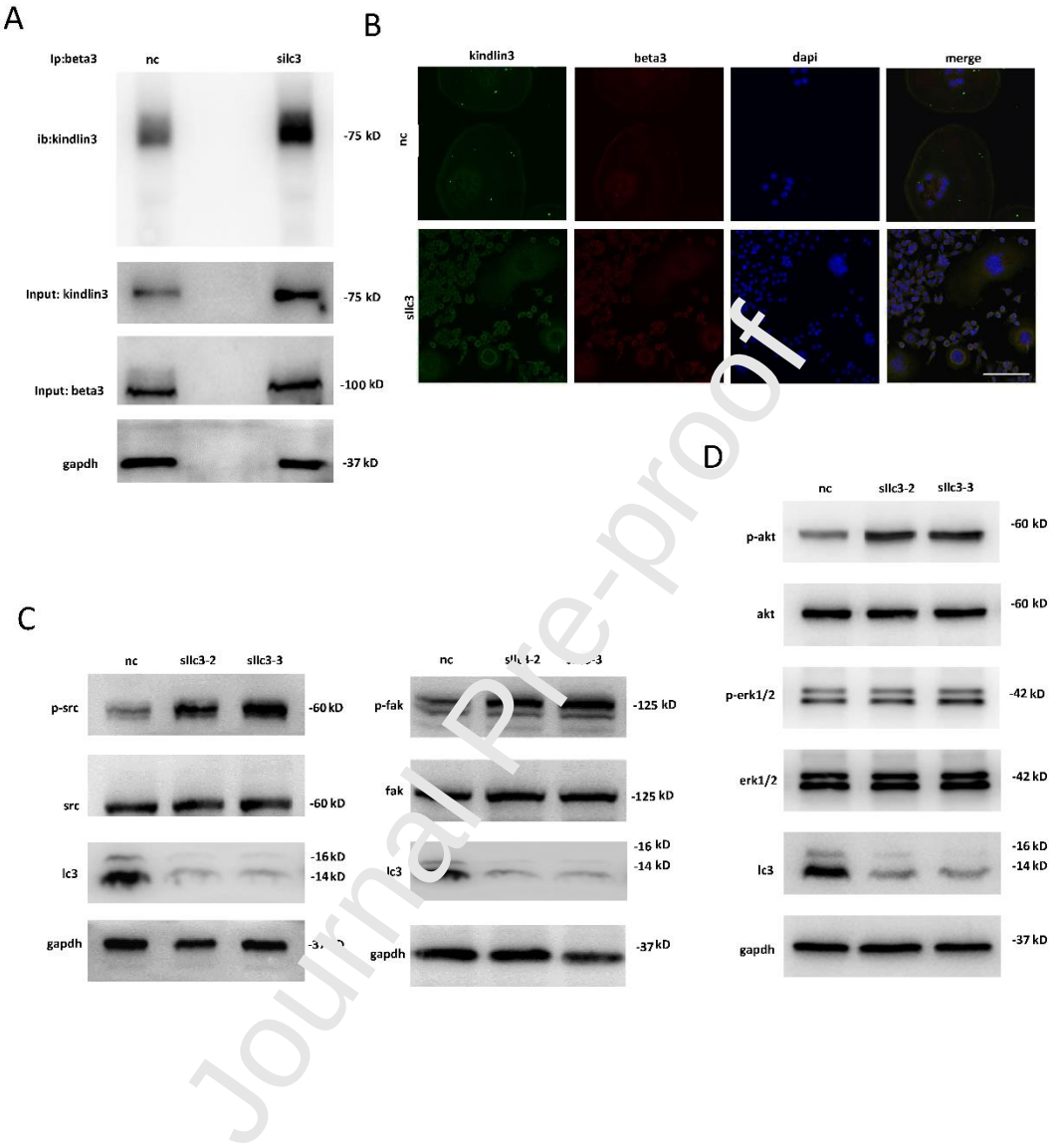


Figure 4 LC3B mediates the integrin/FAK/AKT pathway in osteoclasts

(a) RAW264.7 cells were seeded in 75 cm² culture-flask and treated with RANKL (50 ng/mL) for 4 days. LC3B siRNA intermixture (siLC3B-2 and siLC3B-3) and negative control were transfected into osteoclasts using siRNA Transfection Reagent. Control group osteoclasts and LC3B siRNA-transfected osteoclasts were subjected to immunoprecipitation using a β3 antibody. Kindlin3 binding differences were detected by western blotting.

(b) RAW264.7 cells were seeded in 6-well plates and treated with RANKL (50 ng/mL) for 4 days. LC3B siRNA intermixture (siLC3B-2 and siLC3B-3) and negative control were transfected into osteoclasts. Co-localization of kindlin3 (green) and integrin β3 (red) were detected by immunofluorescence; the nuclei were stained with DAPI; marker bars, 20 μm.

(c, d) After osteoclasts were transfected with LC3B-siRNA, the cells were lysed and subjected to western blot analysis. Levels of total and phosphorylated FAK (Y397), SRC (416), AKT

(Ser473), and ERK1/2 proteins were detected by the respective antibodies. GAPDH was used as a loading control

The above results are representative data of three independent experiments.

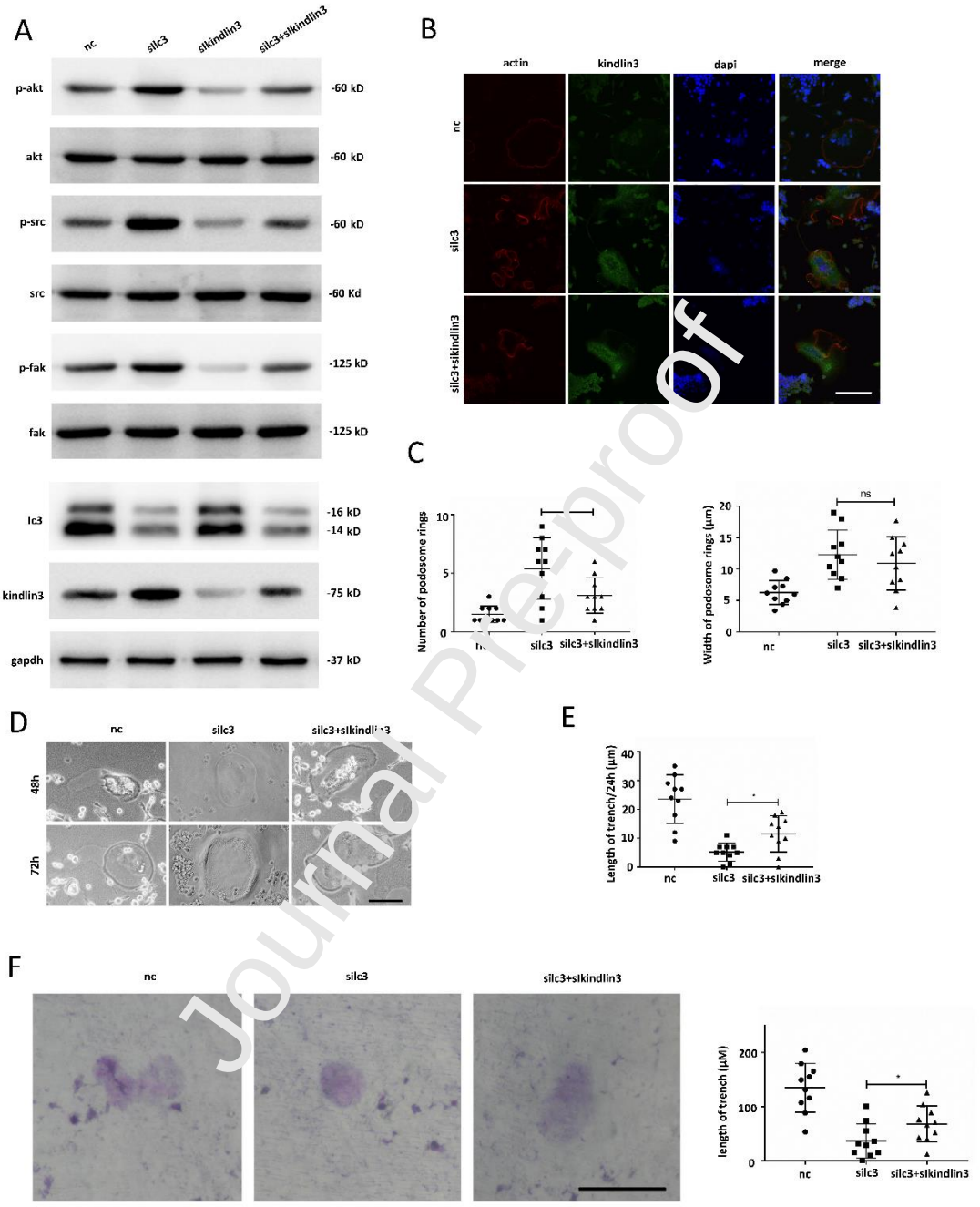


Figure 5 LC3B-mediated autophagy inhibits osteoclast migration via kindlin3

In the experimental group, osteoclasts were transfected with LC3B or kindlin3 siRNA. In the recovery group, osteoclasts were transfected both LC3B and kindlin3 siRNA at the same time. SiRNA-NC was used as a negative control.

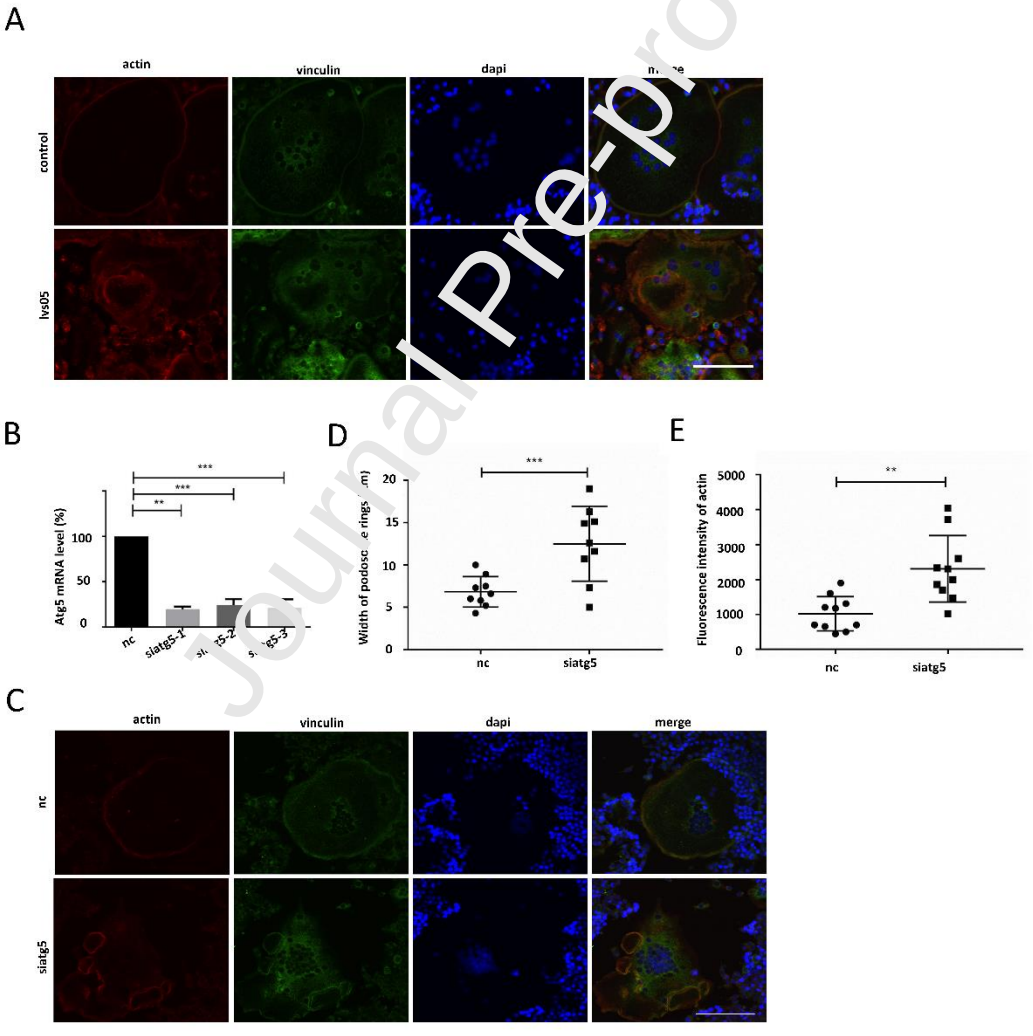
(a) SiRNAs were transfected into osteoclasts using siRNA Transfection Reagent for 48 h.

The proteins were then separated by SDS-PAGE; levels of total and phosphorylated SRC/FAK/AKT were detected by each primary antibody.

(b, c) The change of kindlin3 (green) and actin (red) were detected by immunofluorescence; nuclei were stained with DAPI; marker bars, 100 μm. The number and width of podosome rings were analyzed by Cellsens Dimension. Data are presented as means ± SD. *P<0.05, ns P>0.05

(d, e) Osteoclasts were seeded on calcium phosphate-coated plates, and the migration distance was measured at each time point by ImageJ, and the difference between the two measurements was reported as the distance osteoclasts migrated in 24 h; Data are presented as means ± SD. n = 10. *P<0.05

(f) Osteoclasts were transfected with LC3B or kindlin3 siRNA, and the migration distance of osteoclasts on the dentin was stained by toluidine blue and measured by ImageJ; marker bars, 100 μm. Data are presented as means ± SD. n = 10. *P<0.05



Supplemental Figure. 1

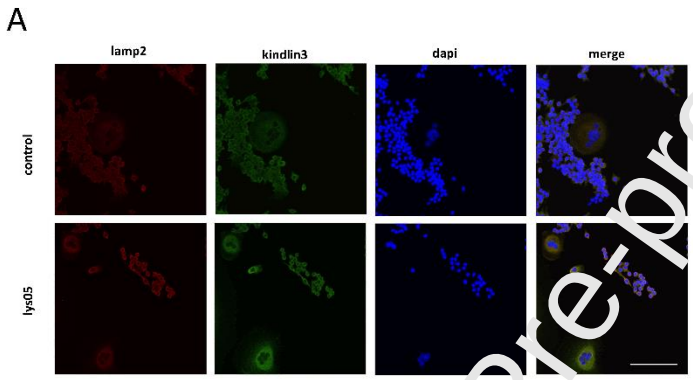
(a)Osteoclasts from Bone Marrow Cells were treated with 2μM Lys05 for 48h; podosome

rings are stained for actin (red) and vinculin (green); the nuclei were stained with DAPI; marker bars, 100 μm. The results shown are representative data of three independent experiments.

(b)Three different kinds of ATG5 siRNA were transferred into osteoclasts, respectively. After 48 h, the total RNA was extracted from lysed cells, and the expression of ATG5 was measured by quantitative RT-PCR. Data are presented as means ± SD. **P < 0.01,***P<0.001.

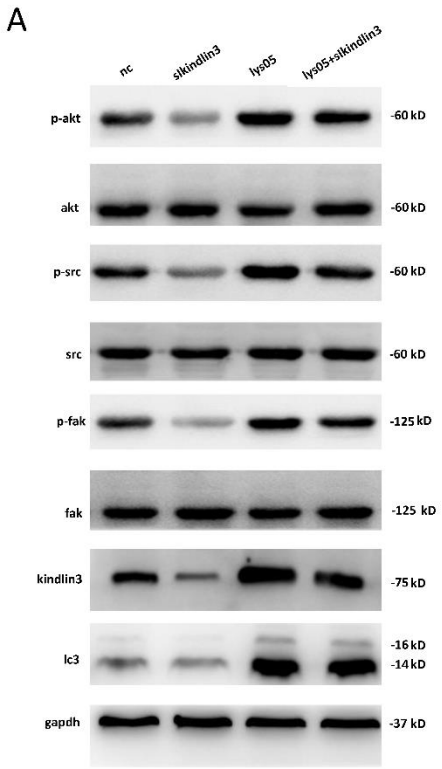
(c) Osteoclast was transfected with ATG5 siRNA intermixture or negative control for 48 h; podosome rings are stained for actin (red) and vinculin (green); the nuclei were stained with DAPI; marker bars, 100 μm.

(d,e) The fluorescence intensity of actin and the width of podosome rings were measured by cellSens Dimension. Data are presented as mean ± SD. n = 10. **P <0.01, ***P<0.001.



Supplemental Figure. 2

(a) Osteoclasts were incubated with or without Lys05 (2μM) in the presence of RANKL (50 ng/ml). Co-immunostaining analysis of kindlin3 (green) and lamp2 (red) distribution in mature osteoclasts; the nuclei were stained with DAPI; marker bars, 100 μm. Results are representative data of three independent experiments



Supplemental Fig. 3

(a)In the experimental group, osteoclasts were transfected with kindlin3 siRNA or treated with Lys05. In the recovery group, osteoclasts were treated with kindlin3 siRNA and Lys05 at the same time. SiRNA-NC was used as a negative control. SiRNAs were transfected into osteoclasts using siRNA Transfection Reagent for 48 h. The proteins were then separated by SDS-PAGE; levels of total and phosphorylated SRC/FAK/AKT were detected by each primary antibody. The results shown are representative data of three independent experiments.

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Yuang Zhang: Data curation, Writing

Highlights

1. A novel interaction between LC3B and kindlin3 in osteoclasts was discovered.
2. LC3B can regulate the activation of integrin β3 through kindlin3 in osteoclasts.
3. LC3B can affect osteoclast migration by regulating podosome degradation and may represent a new treatment for bone-related diseases caused by the dysfunction of osteoclasts.