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Lysosomal pH Plays a Key Role in Regulation of mTOR Activity in Osteoclasts

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ABSTRACT

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase involved in the regulation of cell growth. It has been shown to play an important role in osteoclast differentiation, particularly at the earlier stages of osteoclastogenesis. mTOR activation and function, as part of mTORC1 complex, is dependent on lysosomal localization and the vacuolar H^+-ATPase (V-ATPase) activity; however, the precise mechanism is still not well understood. Using primary mouse osteoclasts that are known to have higher lysosomal pH due to R740S mutation in the V-ATPase *a3* subunit, we investigated the role of lysosomal pH in mTORC1 signaling. Our results demonstrated that +/R740S cells had increased basal mTOR protein levels and mTORC1 activity compared to +/+ osteoclasts, while mTOR gene expression was decreased. Treatment with lysosomal inhibitors chloroquine and ammonium chloride, compounds known to raise lysosomal pH, significantly increased mTOR protein levels in +/+ cells, confirming the importance of lysosomal pH in mTOR signaling. These results also suggested that mTOR could be degraded in the lysosome. To test this hypothesis, we cultured osteoclasts with chloroquine or proteasomal inhibitor MG132. Both chloroquine and MG132 increased mTOR and p-mTOR protein levels in +/+ osteoclasts, suggesting that mTOR undergoes both lysosomal and proteasomal degradation. Treatment with cycloheximide, an inhibitor of new protein synthesis, confirmed that mTOR is constitutively expressed and degraded. These results show that, in osteoclasts, the lysosome plays a key role not only in mTOR activation but also in its deactivation through protein degradation, representing a novel molecular mechanism of mTOR regulation. J. Cell. Biochem. 117: 413–425, 2016.

KEY WORDS: OSTEOCLAST; V-ATPase; LYSOSOME; pH; mTOR; AUTOPHAGY

Osteoclasts are bone resorbing multinucleated cells formed by fusion of mononuclear precursors of hematopoietic origin [Boyle et al., 2003]. Two molecules, macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL), play key roles in osteoclastogenesis, leading to activation of transcription factors and expression of proteins necessary for osteoclast function [Boyle et al., 2003; Takayanagi, 2007].

Serine/threonine kinase mammalian target of rapamycin (mTOR) has been shown to play an important role in osteoclast differentiation; it is activated by M-CSF and its inhibition leads to decreased osteoclastogenesis [Glantschnig et al., 2003; Indo et al., 2013]. Furthermore, mTOR expression levels are higher at the earlier stages of osteoclastogenesis and decrease at the later stages of osteoclast formation [Indo et al., 2013]. mTOR is ubiquitously expressed and plays key role in cell growth, proliferation, and differentiation in response to nutrient availability and growth factor signaling [Betz and Hall, 2013]. In cells, mTOR exists as part of two complexes: complex 1 (mTORC1) and complex 2 (mTORC2). These

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complexes have different functions: mTORC2 primarily is being involved in growth factor signaling, while mTORC1 is activated by amino acids, growth factors, oxygen, inflammation, and Wnt signaling [Laplante and Sabatini, 2013]. mTORC1 is also a negative regulator of autophagy, a lysosomal degradation process responsible for removal of long-lived proteins and damaged organelles [Mizushima and Komatsu, 2011; Klionsky et al., 2012]. Macroautophagy (hereafter referred to as autophagy) is a tightly regulated process, characterized by formation of an autophagosomal membrane around target proteins, and engulfing them. mTORC1 inhibits autophagy directly, by blocking formation of the autophagosomal membrane. The autophagosomes later fuse with the lysosomes to form autolysosomes, leading to degradation of the target proteins.

Lysosomes are highly acidic (pH ~4.5) intracellular organelles and their primary function is degradation of extracellular and intracellular material [Appelqvist et al., 2013; Settembre et al., 2013]. The molecule responsible for maintaining such low lysosomal pH is the vacuolar H^+- ATPase (V-ATPase), a ubiquitous multi-subunit proton pump [Forgac, 2007; Maxson and Grinstein, 2014]. Lysosomes play an integral role in autophagy, and lysosomal inhibitors, such as ammonium chloride and chloroquine, and the V-ATPase inhibitors, such as bafilomycin A and concanamycin A, are commonly used as autophagy inhibitors [Klionsky et al., 2012].

Prior studies have suggested a connection between the lysosome, V-ATPases, and mTOR (for a review of this topic, see [Efeyan et al., 2012]). It has been shown that upon amino acid signaling, mTORC1 is translocated to the lysosomal membrane where it interacts with the Ragulator complex. A direct interaction between the Ragulator complex and several subunits of the V-ATPase has been shown [Sancak et al., 2010; Bar-Peled et al., 2012]. The Ragulator complex serves as a scaffold for the Rag GTPases, a group of GTPases shown to recruit and activate mTORC1. The precise mechanism of mTORC1 activation by amino acids is still not clear; however, it is known that amino acid signaling is (1) initiated inside the lysosome, (2) requires V-ATPase activity, and (3) is described as "inside-out model of amino acid sensing" [Zoncu et al., 2011; Laplante and Sabatini, 2013]. As V-ATPases appear to play one of the key roles, we decided to investigate the role of the V-ATPases and lysosomal pH in regulation of mTORC1 activation and function.

To elucidate the role of lysosomal pH, we used a mouse model with a point mutation in the V-ATPase *a*3 subunit [Ochotny et al., 2011; Voronov et al., 2013]. In this model, an evolutionary conserved arginine is replaced with serine (R740S), resulting in a V-ATPase complex that is properly targeted to lysosomes but with diminished proton-pumping activity. Heterozygous (+/R740S) animals have mild osteopetrosis, while homozygous mice develop severe disease [Ochotny et al., 2013]. V-ATPases containing the *a*3 subunit are highly enriched in osteoclasts [Li et al., 1999; Toyomura et al., 2000; Manolson et al., 2003]. In actively resorbing osteoclasts, V-ATPases are targeted to the ruffled border, a portion of the convoluted plasma membrane adjacent to the bone surface during active bone resorption. In vitro, in non-resorbing cells, V-ATPases containing *a*3 subunit are localized to lysosomes and plasma membranes of the osteoclasts [Toyomura et al., 2003]. We previously demonstrated that osteoclasts from +/R740S mice have increased lysosomal pH [Voronov et al., 2013]. Therefore, the cells with R740S mutation are an ideal tool to elucidate the role of lysosomal pH and the V-ATPases in mTORC1 signaling in osteoclasts. Here, we show for the first time that in primary osteoclasts, in addition to amino acids and growth factor signaling, lysosomal degradation of mTOR is another regulator of its activity, further demonstrating the importance of lysosomal pH in intracellular signaling.

MATERIALS AND METHODS

ANIMALS

Heterozygous mice carrying the R740S mutation bred onto the C3H/HeJ and FVB backgrounds were generated as described previously [Ochotny et al., 2011]; +/+ and +/R740S littermates were used in experiments described below. All experimental procedures received approval from the local Animal Care Committees and were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

OSTEOCLAST CULTURES

Bone marrow (BM)-derived mononuclear cells from femurs of 2-month-old male mice were plated at a cell density of 1×10^5 cells/ml in 10% FBS in alpha-MEM (Gibco/Life Technologies) and antibiotics (10 μg/ml penicillin G, 50 μg/ml gentamycin, 0.03 μg/ml fungizone (Gibco/Life Technologies) supplemented with 25 ng/ml M-CSF (R&D Systems) for 2 days, and then with 25 ng/ml M-CSF and 100 ng/ml RANKL (made in-house) for another 4 days at 37°C in 5% CO_2. To monitor osteoclastogenesis, tartrate-resistant acid phosphatase (TRAP) staining was performed with every experiment. For TRAP staining, the cells were cultured in 96-well plates as previously described, briefly fixed in formalin, stained with a solution consisting of 50 mM acetate buffer, 30 mM sodium tartrate, 0.1 mg/ml naphthol AS-MX phosphate, 0.1% Triton×-100, and 0.3 mg/ml Fast Red Violet LB for 10–20 min, and then washed three times with dH_2O. TRAP-positive cells containing five or more nuclei were counted.

STARVATION AND RECOVERY EXPERIMENTS

BM-derived osteoclasts were cultured as described above. For starvation experiments, on the last day of the culture, the cells were washed two times with warm Hank's balanced salt solution (HBSS, Gibco/Life Technologies) to remove traces of amino acids and serum, and then incubated at 37°C in 5% CO_2 for specified time periods. The cells were then either fixed for immunofluorescence experiments or lysed for protein analysis (see below). For recovery experiments, the cells were washed two times with warm HBSS, incubated with HBSS for 1 h at 37°C in 5% CO_2 and then treated with fully supplemented media containing 12.5 ng/ml M-CSF and 50 ng/ml RANKL for additional 30 min. Cells were then lysed for protein analysis, as described below.

mTOR GENE EXPRESSION

On day 6 of osteoclast culture, total RNA was extracted using Trizol reagent (Invitrogen). For quantitative real time PCR (qRT-PCR) gene expression analysis, cDNA was synthesized using SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to the manufacturer's

instructions. qRT-PCR was performed using TaqMan Universal Master Mix II with UNG (cat# 4440042, Applied Biosystems) and TaqMan primer-probe mix (mTOR cat.# Mm 00444968-m1, 18S cat. # Mm 03928990-g1) in StepOnePlus Real-Time PCR System (Applied Biosystems, Life Technologies). All data were normalized to expression of 18S, a ribosomal RNA. The experiments were repeated four times.

LYSOSOMAL AND mTOR INHIBITORS

BM-derived osteoclasts were cultured as described above. On day 5 of culture, medium was replaced with one containing 12.5 ng/ml M-CSF, 50 ng/ml RANKL, and inhibitors (ammonium chloride or chloroquine [Sigma–Aldrich], rapamycin [Cayman Chemicals]). Cells were incubated for indicated time periods and then used either for protein analysis or immunofluorescence, as described below.

IMMUNOBLOTTING

BM-derived osteoclasts were cultured in 6-well tissue culture plates as described earlier. The plates were washed two times with cold PBS and lysed using RIPA buffer (0.1% triton×-100, 50 mM Tris, 300 mM NaCl, 5 mM EDTA), containing protease inhibitors (protease inhibitors cocktail P8340 [Sigma–Aldrich] and 1 mM PMSF), and phosphatase inhibitors (Sigma–Aldrich, P5726). Whole cell lysates were separated on 4–20% gradient TGX gels (BioRad), transferred to nitrocellulose membrane and probed for LC3B, p62, mTOR, p-mTOR, p-4E-BP1, and actin (all from Cell Signaling Technologies [CST]). Amersham™ ECL™ Prime Western blotting reagent was used as a detection reagent. Images were captured using BioRad ChemiDoc Gel Docking system and analyzed by rolling disk method using Quantity One software (BioRad). All experiments were repeated at least three times.

CONFOCAL MICROSCOPY

BM-derived osteoclasts were cultured as described above. For LC3 staining (CST, #2775), cells were fixed for 30 min at 37°C in 4% paraformaldehyde in 80 mM Hepes (pH 7.2) with 6.8% sucrose and permeabilized cold methanol for 10 min as per manufacturer's instructions. For mTOR (CST, #2983) and LAMP2 (Developmental Studies Hybridoma Bank, Iowa, #ABL-93c) staining, cells were fixed for 30 min at 37°C in 4% paraformaldehyde in 80 mM Hepes (pH 7.2) with 6.8% sucrose, permeabilized/blocked for 1 h in 0.3% Triton-×, 5% normal goat serum (NGS) and 1% BSA in PBS. The samples were then incubated with the primary antibodies, anti-mTOR, anti-LC3 (CST, 1:200 and 1:400, respectively), and anti-LAMP2 (1:400), in 1% BSA, 1% NGS, and 0.3% Triton-× in PBS for 16 h, and then with secondary anti-rabbit Alexa Fluor 555 (CST, #4413) or anti-rat Alexa Fluor 488 (CST, #4416) antibody for 1 h; the slides were mounted in DAPI (4′6-diamidino-2-phenylindole)-containing mounting media (ProLong®Gold antifade reagent, Life Technologies). The samples were analyzed with a 63 × 1.35 NA oil-immersion lens using a spinning-disk confocal microscope (Olympus IX81) equipped with an EM-CCD camera (C9100-13, Hamamatsu, Quorum Technologies, Inc.) under control of Volocity software (Perkin-Elmer, Waltham, MA).

CYCLOHEXIMIDE PROTEIN DEGRADATION ASSAY

BM-derived osteoclasts were cultured as described above. On day 6 of culture, medium was replaced with one containing 12.5 ng/ml M-CSF, 50 ng/ml RANKL, and 10 μg/ml cycloheximide (CHX) or DMSO (vehicle control). The cells were incubated for 0, 1, 2, and 4 h. At the end of the incubation period, total protein was collected as described in "Immunoblotting" Section. Protein concentration was measured using Pierce 660 nm protein assay kit, and 20 μg of protein per lane was loaded on 4–15% gradient gel (BioRad). Blots were probed with mTOR and actin antibodies as described in "Immunoblotting" Section. The experiments were repeated four times.

STATISTICAL ANALYSIS

To analyze combined data of all immunoblotting experiments (except CHX), the SAS general linear model (GLM) procedure was used to carry out an analysis of variance to estimate the effect of genotype and factors such as protein type and time and their interactions. An analysis of residuals led to the adoption of the natural logarithm of the response which reduced their skewness and improved the model R squares. When several comparisons were made to a control the *P*-values were adjusted using the Dunnett method. Sample size is as indicated in the methods and figures; *P*-values were considered significant at $P < 0.05$.

RESULTS

AUTOPHAGY IS BLOCKED, WHILE mTORC1 IS MORE ACTIVE IN +/R740S OSTEOCLASTS

We previously demonstrated that +/R740S osteoclasts have higher lysosomal pH compared to +/+ cells (pH 5.71 ± 0.44 *vs.* 4.85 ± 0.18 in +/R740S and +/+, respectively) [Voronov et al., 2013]; however, the mTOR signaling and autophagy were not assessed in these cells. As lysosomal pH is increased in +/R740S cells, it is very likely that autophagy, a protein degradation process dependent on proper lysosomal function, is impaired in +/R740S cells. Autophagy is a dynamic process, therefore, to assess the state of autophagy in +/R740S cells, we looked at protein expression levels of the autophagy markers LC3 and sequestosome 1 (SQSTM1 or p62) at several time points using immunoblotting. LC3-I is commonly used as a marker of autophagosome formation; it is a cytosolic protein which becomes lipidated (LC3-II) upon induction of autophagy, and incorporated into autophagosomal membranes [Klionsky et al., 2012]. As autophagy proceeds, LC3-II is degraded and its levels decrease with time. p62 is a chaperone and an autophagy substrate, and it also gets degraded during autophagy; thus, it is commonly used as a marker of autophagic degradation. Since autophagy is activated by starvation (amino acid and growth factor depletion), in order to assess the autophagic flux in osteoclasts, we incubated the cells with HBSS for 30, 60, and 120 min. At all time points tested, +/R740S osteoclasts had significantly higher levels of LC3-II compared to +/+ controls (Fig. 1A and B); the levels of p62 were also significantly increased in +/R740S cells. Immunofluorescence experiments confirmed that +/R740S cells had higher basal levels of LC3 puncta compared to +/+ cells (Fig. 1C). Accumulation of LC3-II and p62 could be due to either increased autophagy initiation or block in autophagy degradation [Klionsky et al., 2012]. Since our cells have high lysosomal pH, this LC3- II and p62 accumulation was likely due to blocked autophagic degradation. At the same time,

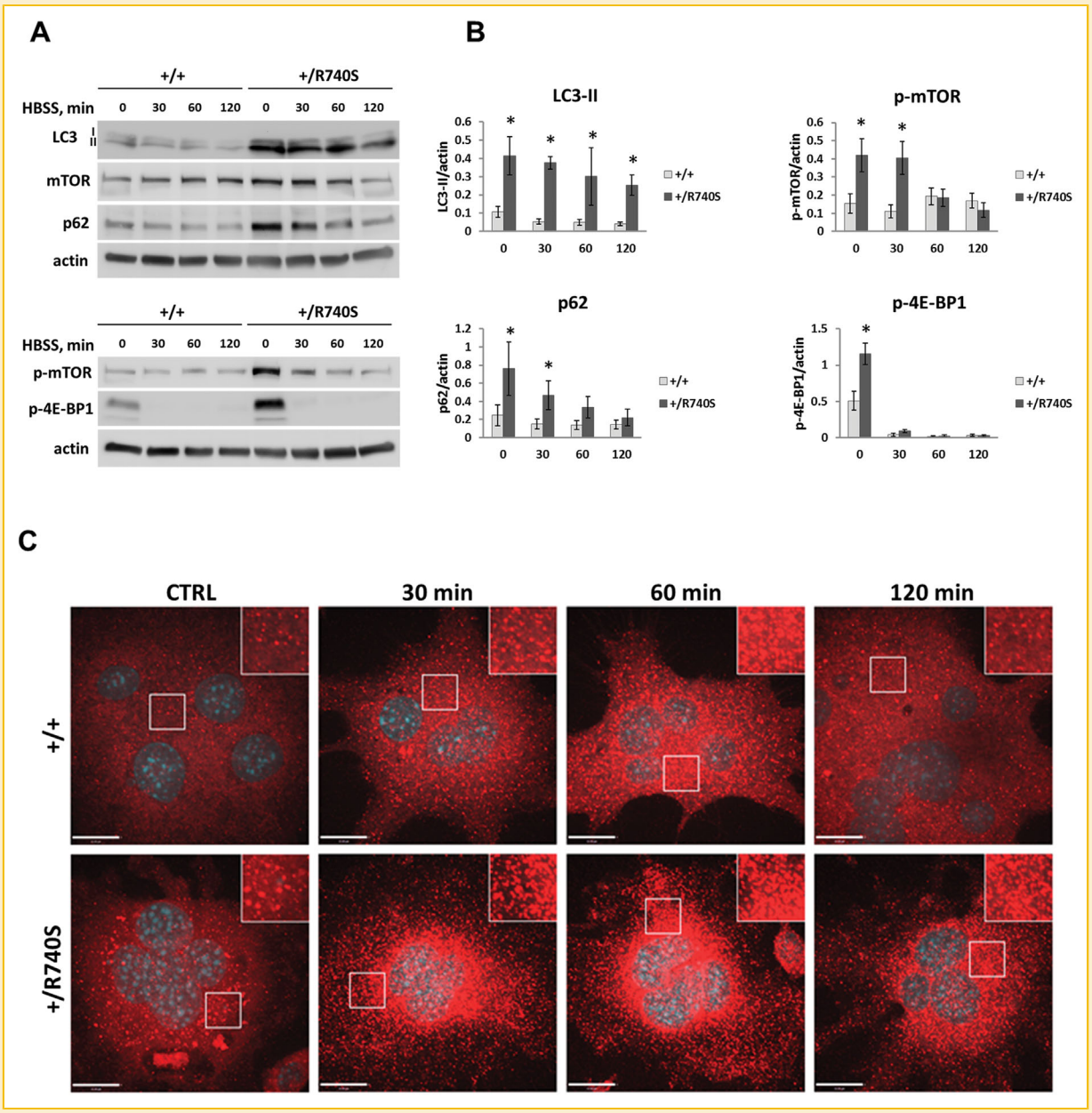

Fig. 1. Autophagy is impaired in +/R740S osteoclasts. Bone marrow-derived osteoclasts were differentiated in the presence of 25 ng/ml M-CSF and 100 ng/ml RANKL. On day 5 of culture, the cells were incubated with HBSS (starvation) for 30, 60, or 120 min. Whole cell lysates were separated on 4–20% gradient gels, transferred to a nitrocellulose membrane, and probed for LC3, p62, mTOR, p-mTOR, and actin. (A) Representative blots. (B) Quantification of immunoblots; combined data of four independent experiments, geometric mean ± SEM; * indicates statistical significance compared to +/+ control, $P < 0.05$. (C) LC3 immunostaining. BM-derived osteoclasts were incubated with HBSS for the indicated time periods, fixed, stained, and analyzed by confocal microscopy as described in "Methods." Representative images. Bars, 15 μm.

since the levels of both LC3-II and p62 did decrease with time in +/R740S cells, it appeared that the autophagy was not completely blocked, but rather may have had a decreased rate of cargo/target protein degradation.

Next, we evaluated the effect of higher lysosomal pH on mTORC1 activity. To assess whether mTORC1 function was affected in +/R740S osteoclasts, we looked at the protein levels of mTOR, p-mTOR, as well as mTORC1 substrate eukaryotic translation initiation factor 4E binding protein 1 (p-4E-BP1) by immunoblotting (Fig. 1A and B). The baseline levels of mTOR, p-mTOR, and p-4E-BP1 were significantly higher in +/R740S compared to +/+ cells. This was unexpected, since increased mTOR activity is usually associated with decreased autophagy. At the same time, amino acid withdrawal did inhibit mTORC1 activity both in +/+ and +/R740S cells, as seen by decrease in the levels of p-4E-BP1; however, we noticed that while p-mTOR levels

decreased in +/R740S with time, its levels in +/+ osteoclasts did not appear to be significantly affected.

To investigate whether increased mTORC1 activity was due to higher mTOR gene expression, samples were analyzed by q-RT-PCR. mTOR expression was in fact decreased in +/R740S cells compared to +/+ control (Fig. 2A). To check whether mTOR activity was specific to the osteoclasts, the cells were treated with mTORC1 inhibitor rapamycin for the last 24 h of culture and the number of osteoclasts was assessed by TRAP staining (Fig. 2B). As previously reported [Voronov et al., 2013], osteoclastogenesis in +/R740S cultures was decreased. Rapamycin treatment did not appear to affect the formation of osteoclasts in +/+ groups; however, osteoclast number was significantly decreased in +/R740S cells, suggesting that these cells were more sensitive to rapamycin. Therefore, our results indicate that increased baseline mTORC1 activity in +/R740S cells is not caused by higher gene expression but could only be due to changes in lysosomal pH.

mTORC1 ACTIVATION PER SE IS NOT AFFECTED IN +/R740S CELLS

To elucidate the cause of increased mTOR protein levels and mTORC1 activation in +/R740S osteoclasts, we investigated whether mTORC1 activation *per se* was affected in +/R740S osteoclasts. Since mTORC1 is activated by amino acids and growth factors, the cells were deprived of amino acids and serum for 1 h (HBSS, starvation) and then incubated with fully supplemented media containing M-CSF (12.5 ng/ml) and RANKL (50 ng/ml) for additional 30 min (recovery). As previously observed, +/R740S cells had higher baseline protein levels of mTOR, p-mTOR and p-4E-BP1 compared to +/+ cells (Fig. 3A and B); however, in response to starvation and upon recovery, there was no difference in p-mTOR and p-4E-BP1 protein levels between +/+ and +/R740S cells (Fig. 3A and B), suggesting that mTORC1 activity per se in response to starvation and stimulation (amino acids, serum, and M-CSF) was not affected in +/R740S cells; however, these results did not explain why at baseline conditions +/R740S osteoclasts have higher mTORC1 activity compared to +/+ cells, as measured by p-4E-BP1 and p-mTOR protein levels.

mTOR PROTEIN LEVELS INCREASE IN THE PRESENCE OF LYSOSOMAL INHIBITORS

To investigate the mechanism of increased mTOR and p-mTOR levels in +/R740S cells and to mimic the conditions observed in +/R740S cells, osteoclasts were treated with lysosomal inhibitors chloroquine (CHQ) and ammonium chloride (NH_4Cl), both compounds known to increase lysosomal pH, for 18 h. As expected, this treatment inhibited autophagy in +/+ osteoclasts, as seen by increased LC3-II protein levels, but had no effect on LC3-II levels in +/R740S cells as measured by immunoblotting (Fig. 4A and B). Treatment of +/+ osteoclasts, but not +/R740S cells, with CHQ and NH_4Cl significantly increased mTOR protein levels. The levels of p-mTOR, even though showing a similar trend, failed to reach statistical significance. The levels of p-4E-BP1 were also not affected by the treatment. It is unclear why p-mTOR and p-4E-BP1 levels were not significantly increased, even though mTOR protein levels were higher; these results may reflect the activity of other regulatory mechanisms affecting mTOR, such as dephosphorylation of mTOR by phosphatases [Yu et al., 2008] or modulation of Rag GTPases [Betz and Hall, 2013]. To determine short-term effect of these lysosomal inhibitors, the cells were treated with NH_4Cl and CHQ for 1 h and stained for either LC3 or mTOR. LC3 staining of the cells confirmed that 1 h incubation of the cells with CHQ was sufficient to trigger block of basal autophagy as seen with LC3 puncta accumulation (Fig. 5A). Interestingly, while basal mTOR fluorescence intensity was increased in +/R740S compared to +/+ cells, treatment with lysosomal inhibitors increased it even further (Fig. 5B). Quantification of mTOR fluorescence intensity confirmed a significant increase of mTOR levels both in +/+ and +/R740S cells in response to CHQ, while the effect of NH_4Cl was not as pronounced (Fig. 5C). This slight discrepancy between immunofluorescence and Western blotting results could be explained by the fact that we are comparing image

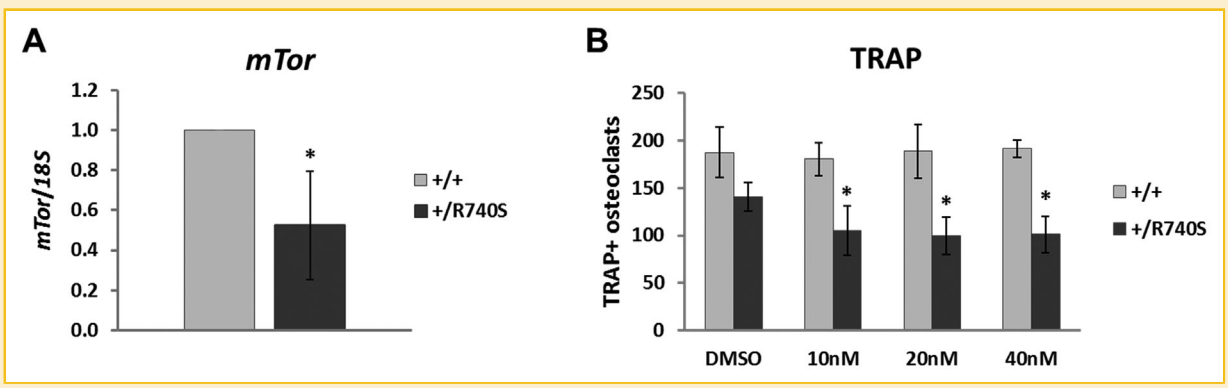

Fig. 2. mTOR gene expression is decreased in +/R740S osteoclasts. Bone marrow-derived osteoclasts were differentiated in the presence of 25 ng/ml M-CSF and 100 ng/ml RANKL. (A) On day 6 of culture, total RNA was collected and mTOR and 18S gene expression was assessed. Combined results of four independent experiments, results are normalized by 18S and are presented as fold change compared to +/+ control, mean ± SD; * indicates statistical significance, $P < 0.05$. (B) On day 5 of culture, the cells were incubated with fully supplemented media containing 12.5 ng/ml M-CSF and 50 ng/ml RANKL and either DMSO (vehicle control) or rapamycin for 24 h. Quantification of TRAP-positive osteoclasts (five or more nuclei). Representative experiment of four independent experiments, mean ± SD; * indicates statistical significance, $P < 0.05$.

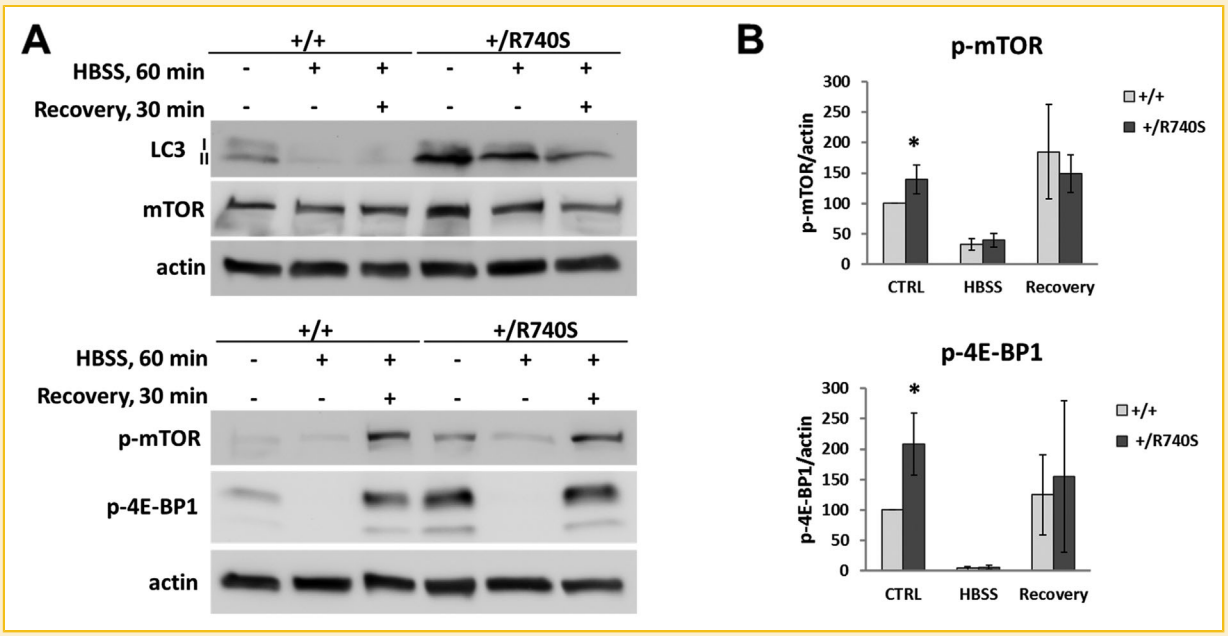

Fig. 3. mTORC1 activation per se is not affected in +/R740S cells. Bone marrow-derived osteoclasts were differentiated in the presence of 25 ng/ml M-CSF and 100 ng/ml RANKL. On day 5 of culture, the cells were incubated with HBSS (starvation) for 60 min and then with fully supplemented media containing 12.5 ng/ml M-CSF and 50 ng/ml RANKL for 30 min. Whole cell lysates were separated on 4–20% gradient gels, transferred to a nitrocellulose membrane and probed for LC3, mTOR, p-mTOR, p-4E-BP1, and actin. (A) Representative blots. (B) Quantification of immunoblots; combined data of four independent experiments expressed as % of +/+ control, mean ± SD; * indicates statistical significance compared to +/+ control, $P < 0.05$.

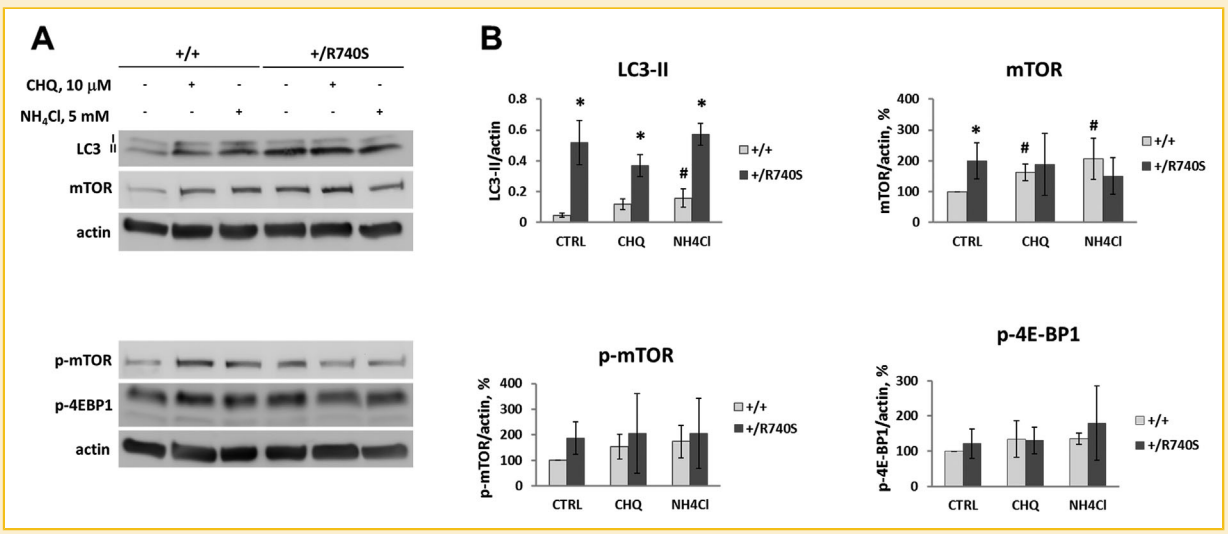

Fig. 4. mTOR protein levels increase in the presence of lysosomal inhibitors. Bone marrow-derived osteoclasts were differentiated in the presence of 25 ng/ml M-CSF and 100 ng/ml RANKL. On day 5 of culture, the cells were treated with NH_4Cl (5 mM) or chloroquine (CHQ, 10 μM) for 24 h in the presence of 12.5 ng/ml M-CSF and 50 ng/ml RANKL. Whole cell lysates were separated on 4–20% gradient gels, transferred to a nitrocellulose membrane, and probed for LC3, mTOR, p-mTOR, p-4E-BP1, and actin. (A) Representative blots. (B) Quantification of immunoblots; combined data of three independent experiments, LC3-II results expressed as geometric mean ± SEM; mTOR, p-mTOR, and p-4E-BP1 results expressed as % of +/+ control, mean ± SD; * indicates statistical significance between +/R740S compared to +/+ control, $P < 0.05$. # indicates statistical significance between +/+ control and +/+ treatment groups, $P < 0.05$.

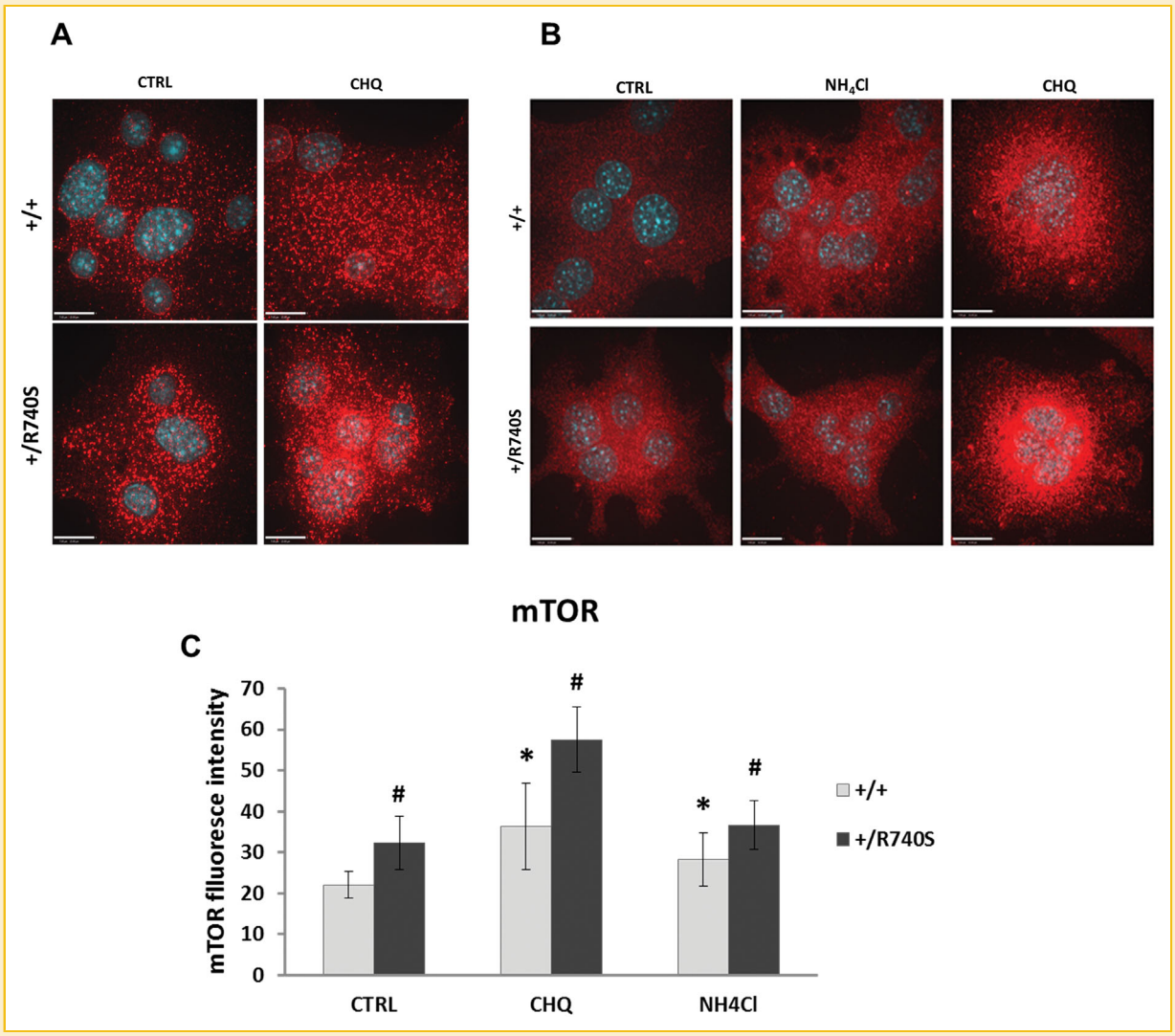

Fig. 5. Fluorescence intensity of mTOR is increased in response to lysosomal inhibitors. Bone marrow-derived osteoclasts were differentiated in the presence of 25 ng/ml M-CSF and 100 ng/ml RANKL. On day 6 of culture, the cells were treated with NH_4Cl (10 mM) or CHQ (20 μM) for 1 h. Cells were fixed, stained, and analyzed by confocal microscopy. Bars, 15 μm. (A) Representative images of osteoclasts stained with anti-LC3B antibody. (B) Representative images of osteoclasts stained with anti-mTOR antibody. (C) Quantification of mTOR immunofluorescence intensity; combined data of two independent experiments, mean ± SD; # indicates statistical significance between +/R740S compared to +/+ control, $P < 0.05$; * indicates statistical significance between +/+ control and +/+ treatment groups, $P < 0.05$.

analysis of single osteoclasts versus analysis of total protein lysate of all the cells present in the cultures. Still, these results suggest that there is an accumulation of mTOR protein during the treatment with lysosomal inhibitors, and this accumulation could be observed after only 1 h of incubation with the inhibitors.

mTOR/LAMP2 CO-LOCALIZATION IS NOT AFFECTED IN +/R740S OSTEOCLASTS

The connection between mTORC1 and the lysosome is well established [Sancak et al., 2010; Zoncu et al., 2011; Efeyan et al., 2012]. It has been shown that active mTORC1 resides on the lysosome, connected to V-ATPases via the Ragulator complex. To verify whether mTOR co-localization with the lysosome is affected in +/R740S cells, we analyzed the cells using confocal microscopy (Fig. 6). Our results showed that in +/+ cells mTOR co-localizes with the endogenous lysosome-associated membrane protein 2 (LAMP2); +/R740S cells display the same levels of co-localization (Pearson's coefficient = 0.72 ± 0.14, n = 13 vs. 0.76 ± 0.078, n = 14, respectively). Furthermore, both NH_4Cl and CHQ treatment (1 h) did not have any effect on mTOR/LAMP2 co-localization (for +/+ cells Pearson's coefficient = 0.67 ± 0.065, n = 7 and 0.71 ± 0.061, n = 10, respectively; for +/R740S cells Pearson's coefficient = 0.72 ± 0.075, n = 10 and 0.72 ± 0.092, n = 12, respectively). These results indicate that changes in lysosomal pH do not affect mTOR-LAMP2 co-localization in osteoclasts and suggest that increased mTORC1 activity in +/R740S cells is not due to improper localization.

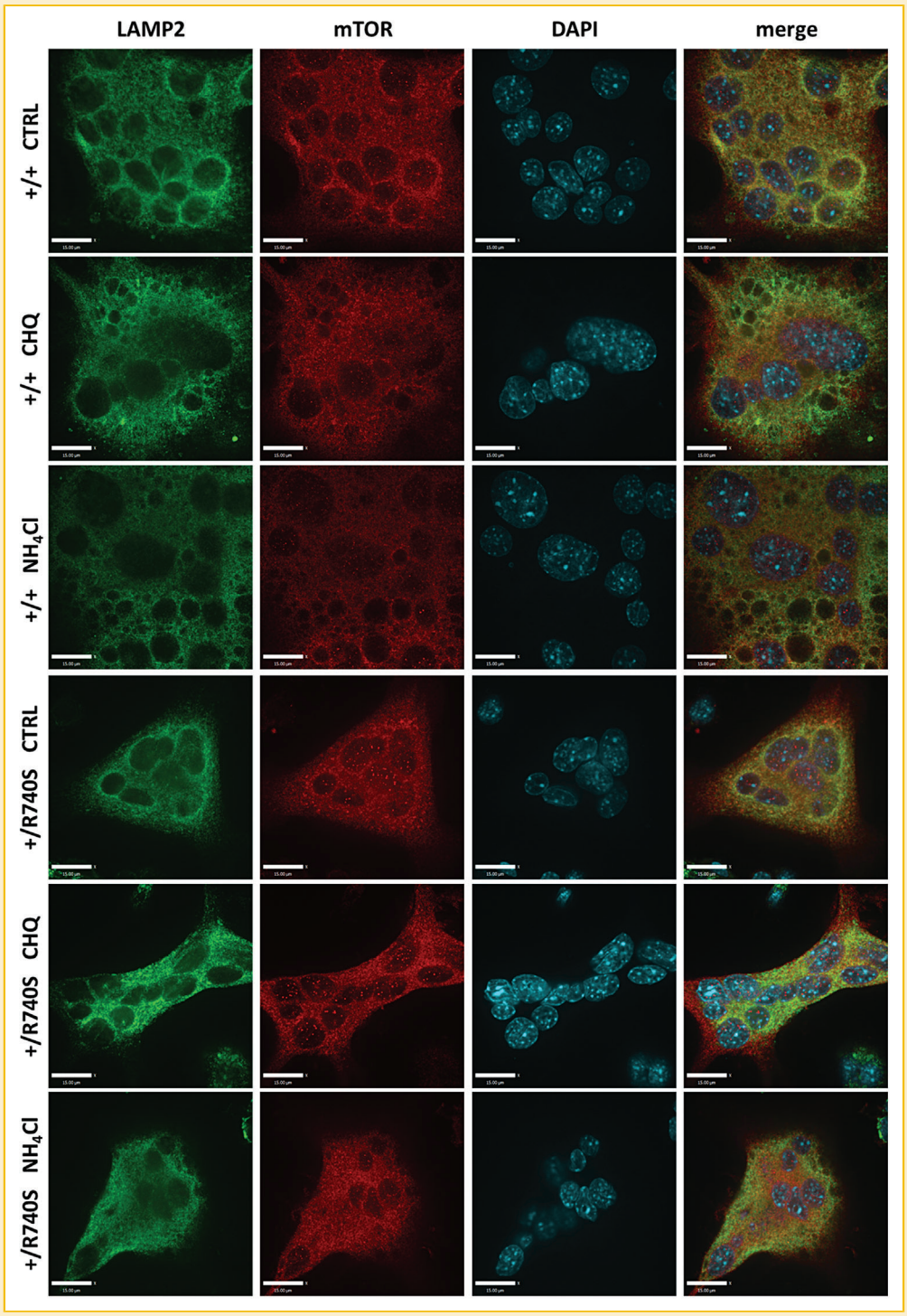

Fig. 6. mTOR/LAMP2 co-localization is not affected in +/R740S osteoclasts. Bone marrow-derived osteoclasts were differentiated in the presence of 25 ng/ml M-CSF and 100 ng/ml RANKL. On day 6 of culture, the cells were treated with NH_4Cl (10 mM) or CHQ (20 μM) for 1 h. Cells were fixed, stained, and analyzed by confocal microscopy. Bars, 15 μm. Representative images of osteoclasts stained with anti-mTOR and anti-LAMP2 antibody.

mTOR IS DEGRADED BOTH IN THE LYSOSOME AND PROTEASOME

Little is known about mTOR degradation. It has been shown that mTOR is ubiquitinated [Linares et al., 2013] suggesting it could be degraded in the proteasome; however, it is not known whether autophagy/lysosomal degradation is involved in regulation of mTOR protein levels. Our results so far suggest that inhibition of lysosomal function leads to mTOR accumulation. To test our hypothesis that mTOR is degraded in the lysosome, we incubated the cells with CHQ, proteasomal inhibitor MG132, and a combination of CHQ and MG132 for 2 h. Results demonstrated that treatment of +/+ cellswith

CHQ or MG132 significantly increased both mTOR and p-mTOR levels; however, there was no cumulative effect on mTOR degradation when both inhibitors were used together (Fig. 7A and B). Furthermore, CHQ had no significant effect on mTOR/p-mTOR levels in +/R740S cells, possibly due to the fact that the lysosomal pH in these cells was already elevated and had no further effect on protein degradation. Interestingly, in +/R740S cells, MG132 decreased mTOR/p-mTOR levels, even though this concentration did not have any effect on cell viability (data not shown). Next, to look at mTOR protein stability, we used cycloheximide. Cycloheximide blocks new protein synthesis; therefore, if mTOR is constitutively degraded, in the absence of new protein synthesis, we expected to observe decreasing amounts of mTOR in the treated samples. We incubated the cells with 10 μg/ml cycloheximide for 1, 2, and 4 h. In +/+, but not in +/R740S cells, mTOR protein levels decreased significantly by 4 h, demonstrating that mTOR is being constitutively degraded in osteoclasts (Fig. 8). This finding is consistent with our hypothesis that mTOR is being degraded via the lysosomal/autophagy pathway, since mTOR protein levels in the +/R740S cells with defective autophagy did not show similar decrease. In summary, our results suggest that lysosomal degradation is one of mechanisms responsible for regulation of mTOR activity and protein levels and, therefore, explain higher baseline levels of mTOR and p-mTOR in +/R740S cells. These results also demonstrate the importance of the lysosome not only in activation of mTORC1 but also in termination of the signal.

DISCUSSION

Autophagy is a lysosomal degradation process necessary for cellular homeostasis, response to stresses (e.g., amino acid starvation), and degradation of long-lived proteins and organelles [Klionsky et al., 2012]. Recent studies have demonstrated that autophagy is also involved in degradation of signaling molecules [Petherick et al., 2013; Jia et al., 2014]. Here, we show for the first time that in osteoclasts, mTOR protein levels and activity are regulated, in part, by lysosomal degradation.

The connection between mTORC1 activity and the lysosome is well established. It has been shown that in the presence of amino acids, active mTORC1 interacts with the V-ATPases located on the lysosomal membrane via the Ragulator complex [Efeyan et al., 2012]. mTOR, as part of mTORC1 complex, is only active when it is associated with the lysosome [Zoncu et al., 2011]. Current hypothesis stipulates that mTOR activation undergoes association and dis-association cycles with the lysosome, depending on free amino acids presence or absence, respectively. There is growing evidence that the lysosome is not only an "activating" component of mTOR pathway but also is involved in its deactivation [Averous et al., 2014; Demetriades et al., 2014]; however, mTOR inactivation as a result of amino acid withdrawal is the only aspect being studied. Here, we considered a possibility that in the presence of amino acids, mTOR is not constitutively active and does not permanently reside on the lysosome but, instead, undergoes constant activation–degradation "cycles" similar to other signaling molecules (e.g., Dishevelled

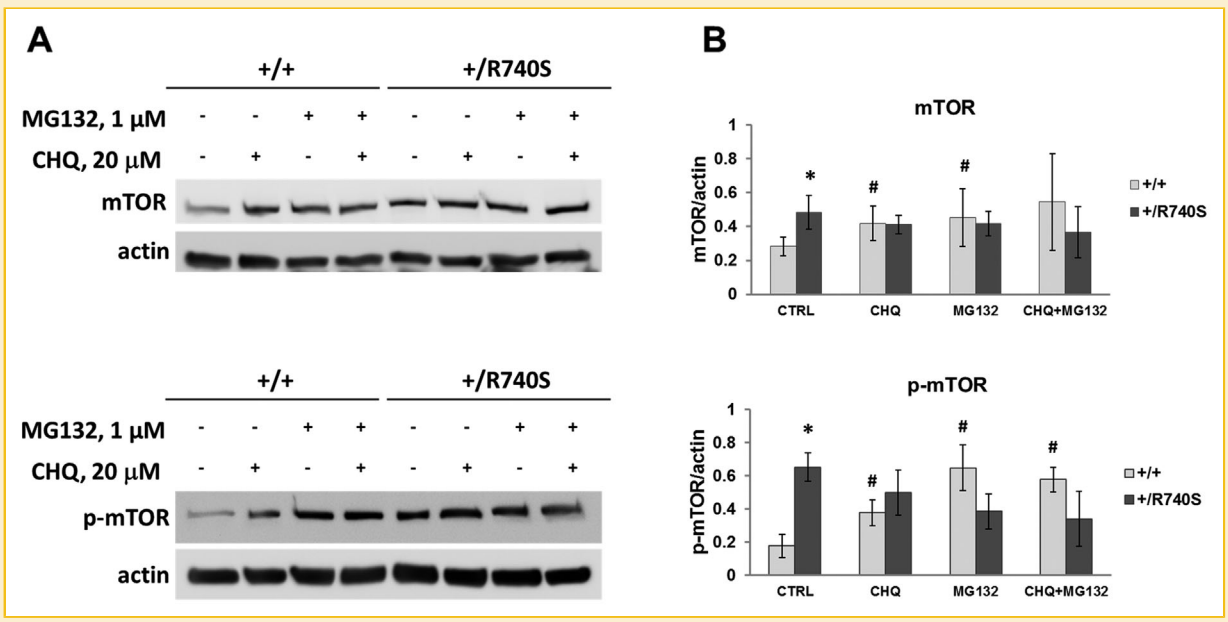

Fig. 7. Both lysosome and proteasome are involved in mTOR degradation. Bone marrow-derived osteoclasts were differentiated in the presence of 25 ng/ml M-CSF and 100 ng/ml RANKL. On day 6 of culture, the cells were treated with 20 μM CHQ, 1 μM MG132, or combination of CHQ and MG132 for 2 h; control group contained corresponding amount of DMSO (vehicle). Whole cell lysates were separated on 4–20% gradient gels, transferred to a nitrocellulose membrane and probed for mTOR, p-mTOR, and actin. (A) Representative blots. (B) Quantification of immunoblots; combined data of five independent experiments, geometric mean ± SEM; * indicates statistical significance between +/R740S compared to +/+ control, $P < 0.05$. # indicates statistical significance between +/+ control and +/+ treatment groups.

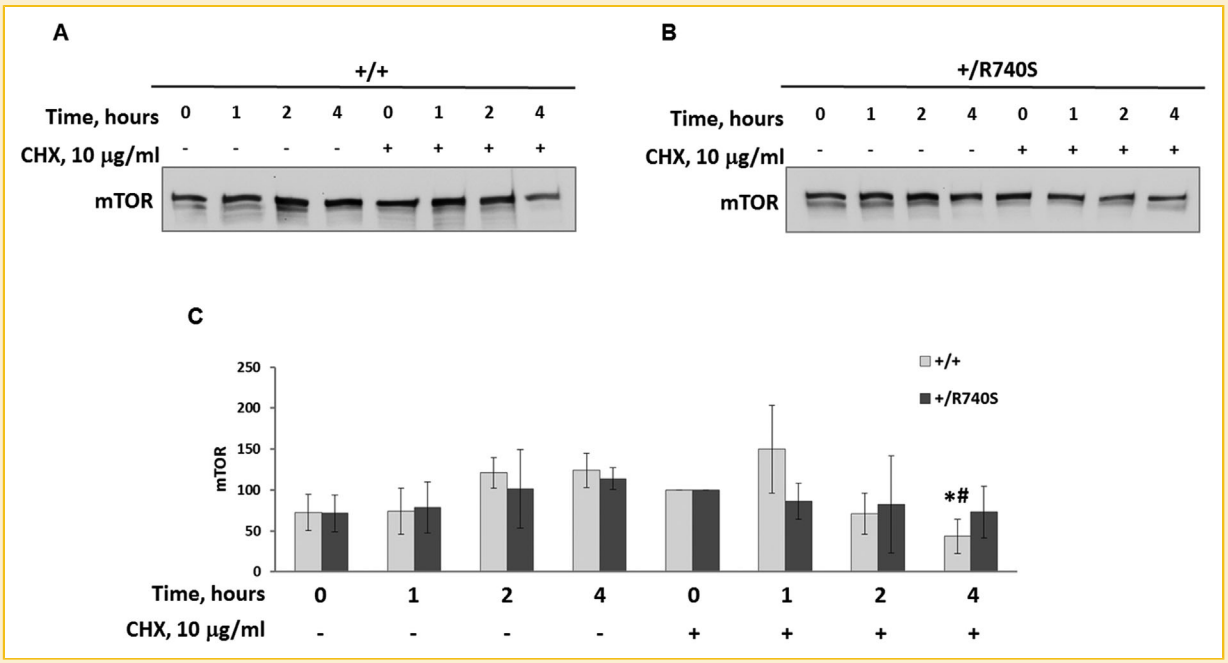

Fig. 8. mTOR is constitutively expressed in osteoclasts. Bone marrow-derived osteoclasts were differentiated in the presence of 25 ng/ml M-CSF and 100 ng/ml RANKL. On day 6 of culture, the cells were treated with 10 μg/ml CHX for 1, 2, or 4 h in fully supplemented media; control groups were treated with media containing corresponding amount of DMSO (vehicle). Whole cell lysates were separated on 4–15% gradient gels, transferred to a nitrocellulose membrane and probed for mTOR. Representative blots (A) +/+ and (B) +/R740S cells. (C) Quantification of immunoblots; combined data of four independent experiments expressed as % mTOR at 0 h CHX treatment, mean ± st dev; * indicates statistical significance between +/+ 4 h CHX compared to +/+ 0 h CHX control, $P < 0.01$. # indicates statistical significance between +/+ 4 h CHX and +/+ 1 h CHX groups, $P < 0.01$ (Student's *t*-test).

degradation in Wnt signaling pathway [Gao et al., 2010] or IκBα degradation in NF-κB canonical pathway [Hayden and Ghosh, 2004; Hayden and Ghosh, 2014]). We hypothesized that mTOR, as part of mTORC1, associates with the lysosome and phosphorylates its target substrates; however, at the end of the "signaling cycle," instead of simply dissociating from the lysosome, it is being degraded in the lysosome in the presence of amino acids to terminate this "cycle." To test our hypothesis, we treated the cells with NH_4Cl and CHQ, both known to increase lysosomal pH and block lysosomal degradation, and showed that both inhibitors increased mTOR protein in +/+ cells. Treatment of +/+ cells with CHQ and proteasomal inhibitor MG132 for 2 h lead to an accumulation of mTOR and p-mTOR, while protein stability experiments using cycloheximide showed a significant decrease in mTOR protein levels in +/+ but not in +/R740S cells. These results support our hypothesis and show that mTOR is constitutively expressed and degraded in the cells in the presence of amino acids, and that both major degradation systems (proteasome and lysosome) are involved in this process.

Little is currently known about mTOR degradation. It has been reported that mTOR is ubiquitinated. In HEK-293T cells, amino acid stimulation promotes the polyubiquitination of mTOR [Linares et al., 2013]. It has also been shown that mTOR undergoes proteasomal degradation, since E3 ubiquitin ligase FBXW7 deletion leads to mTOR accumulation in mouse embryonic fibroblasts from Fbxw7-/- cells [Mao et al., 2008; Fu et al., 2009]. E3 ubiquitin ligases, such as TRAF6 [Linares et al., 2013], Parkin [Park et al., 2014], and CUL-4B-DDB1 [Ghosh et al., 2008] had been demonstrated to associate with mTORC1 complex.

Our experiments with MG132 also confirm that in osteoclasts, mTOR is degraded via the proteasome. The question still remains – is mTOR also degraded in the lysosome? The experiments with CHQ and NH_4Cl seem to suggest that the lysosome is also involved; however, existence of the autophagy-proteasome cross-talk could add more complexity to our results. Inhibition of the proteasomal system activates autophagy [Korolchuk et al., 2010]. At the same time, depending on a cell type, inhibition of autophagy can activate [Wang et al., 2013] or inhibit [Korolchuk et al., 2009] proteasomal activity. In our cells, basal autophagy is inhibited/blocked, leading to accumulation of LC3-II and p62 proteins. Korolchuk et al. [Korolchuk et al., 2009] have shown that in HeLa cells, inhibition of autophagy leads to p62 accumulation, which, in turn, interferes with the clearance of ubiquitinated proteins by the proteasome. At the same time, the activity of the proteasome per se is not affected. It is plausible that in osteoclasts, inhibition of lysosomal function, and consequently of autophagy, either by R740S mutation or by chemical inhibitors (CHQ, NH_4Cl), leads to decreased proteasomal protein degradation, and, therefore, mTOR accumulation. The existence of this cross-talk would also explain why there was no cumulative effect on mTOR protein levels when the cells were treated with both CHQ and MG132.

But how mTOR and lysosomal pH are connected to decreased in vitro osteoclastogenesis in +/R740S cells? Our results show that

+/R740S osteoclasts have higher basal levels of mTOR protein and of mTORC1 activity. mTOR is known to play a role in osteoclastogenesis [Glantschnig et al., 2003] and is more important at the earlier stages of osteoclast differentiation [Indo et al., 2013]. We observed that +/R740S cells not only had higher protein levels of mTOR but also were more sensitive to rapamycin treatment compared to +/+ cells. We believe that this increased sensitivity is due to a delay in osteoclast differentiation in +/R740S cells and the cells remaining at an earlier stage of osteoclastogenesis. This is based on the initial characterization of +/R740S osteoclasts [Voronov et al., 2013], when we observed no difference in gene expression of TRAP, V-ATPase d2 subunit, OSCAR, DC-STAMP, and NFATc1 up to day 4 of osteoclastogenesis; however, expression of all these genes was decreased in +/R740S osteoclasts by days 5 or 6 of culture, possibly indicating a delay in osteoclast differentiation. This delay at the later stages of osteoclastogenesis coincides with *a3* expression; it exponentially increases after day 3 of culture [Toyomura et al., 2000]. Therefore, at earlier stages of osteoclast differentiation, with *a3* being expressed at low levels, there is no differences between +/+ and +/R740S cells; however, at the later stages, when *a3* is expressed at high levels, but is not functional, osteoclastogenesis is impaired. Since *a3* is localized to lysosomes in non-resorbing cells, this delay in differentiation is most likely due to defective degradation of signaling molecules, with mTOR/mTORC1 being one of the signaling pathways affected. Accumulation of active mTORC1 appears to keep the cells in proliferative stage, rather than to allow progression toward differentiation.

In summary, our results indicate that mTOR is degraded in osteoclasts in the presence of amino acids, and both lysosomal and proteasomal degradation systems appear to play a role in this process. We still do not know the precise mechanism of mTOR lysosomal degradation; it is possible that chaperone-mediated autophagy is one of the pathways involved. Nevertheless, here we demonstrate that in osteoclasts, the lysosome plays an important role not only in activation of mTORC1 but also in termination of its activity via lysosomal and proteasomal degradation.

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REFERENCES

Appelqvist H,Waster P, Kagedal K, Ollinger K. 2013. The lysosome: From waste bag to potential therapeutic target. J Mol Cell Biol 5:214–226.

Averous J, Lambert-Langlais S, Carraro V, Gourbeyre O, Parry L, B'Chir W, Muranishi Y, Jousse C, Bruhat A, Maurin AC, Proud CG, Fafournoux P. 2014. Requirement for lysosomal localization of mTOR for its activation differs between leucine and other amino acids. Cell Signal 26:1918–1927.

Bar-Peled L, Schweitzer LD, Zoncu R, Sabatini DM. 2012. Ragulator is a GEF for the rag GTPases that signal amino acid levels to mTORC1. Cell 150:1196–1208.

Betz C, Hall MN. 2013. Where is mTOR and what is it doing there? J Cell Biol 203:563–574.

Boyle WJ, Simonet WS, Lacey DL. 2003. Osteoclast differentiation and activation. Nature 423:337–342.

Demetriades C, Doumpas N, Teleman AA. 2014. Regulation of TORC1 in response to amino acid starvation via lysosomal recruitment of TSC2. Cell 156:786–799.

Efeyan A, Zoncu R, Sabatini DM. 2012. Amino acids and mTORC1: From lysosomes to disease. Trends Mol Med 18:524–533.

Forgac M. 2007. Vacuolar ATPases: Rotary proton pumps in physiology and pathophysiology. Nat Rev Mol Cell Biol 8:917–929.

Fu L, Kim YA, Wang X, Wu X, Yue P, Lonial S, Khuri FR, Sun SY. 2009. Perifosine inhibits mammalian target of rapamycin signaling through facilitating degradation of major components in the mTOR axis and induces autophagy. Cancer Res 69:8967–8976.

Gao C, Cao W, Bao L, Zuo W, Xie G, Cai T, Fu W, Zhang J, Wu W, Zhang X, Chen YG. 2010. Autophagy negatively regulates Wnt signalling by promoting dishevelled degradation. Nat Cell Biol 12:781–790.

Ghosh P, Wu M, Zhang H, Sun H. 2008. MTORC1 signaling requires proteasomal function and the involvement of CUL4-DDB1 ubiquitin E3 ligase. Cell Cycle 7:373–381.

Glantschnig H, Fisher JE, Wesolowski G, Rodan GA, Reszka AA. 2003. M-CSF, TNFalpha and RANK ligand promote osteoclast survival by signaling through mTOR/S6 kinase. Cell Death Differ 10:1165–1177.

Hayden MS, Ghosh S. 2004. Signaling to NF-kappaB. Genes Dev 18:2195–2224.

Hayden MS, Ghosh S. 2014. Regulation of NF-kappaB by TNF family cytokines. Semin Immunol 26:253–266.

Indo Y, Takeshita S, Ishii KA, Hoshii T, Aburatani H, Hirao A, Ikeda K. 2013. Metabolic regulation of osteoclast differentiation and function. J Bone Miner Res 28:2392–2399.

Jia Z, Wang J, Wang W, Tian Y, XiangWei W, Chen P, Ma K, Zhou C. 2014. Autophagy eliminates cytoplasmic beta-catenin and NICD to promote the cardiac differentiation of P19CL6 cells. Cell Signal 26(11):2299–2305.

Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, Agholme L, Agnello M, Agostinis P, Aguirre-Ghiso JA, Ahn HJ, Ait-Mohamed O, Ait-Si-Ali S, Akematsu T, Akira S, Al-Younes HM, Al-Zeer MA, Albert ML, Albin RL, Alegre-Abarrategui J, Aleo MF, Alirezaei M, Almasan A, Almonte-Becerril M, Amano A, Amaravadi R, Amarnath S, Amer AO, Andrieu-Abadie N, Anantharam V, Ann DK, Anoopkumar-Dukie S, Aoki H, Apostolova N, Arancia G, Aris JP, Asanuma K, Asare NY, Ashida H, Askanas V, Askew DS, Auberger P, Baba M, Backues SK, Baehrecke EH, Bahr BA, Bai XY, Bailly Y, Baiocchi R, Baldini G, Balduini W, Ballabio A, Bamber BA, Bampton ET, Banhegyi G, Bartholomew CR, Bassham DC, Bast RCJr, Batoko H, Bay BH, Beau I, Bechet DM, Begley TJ, Behl C, Behrends C, Bekri S, Bellaire B, Bendall LJ, Benetti L, Berliocchi L, Bernardi H, Bernassola F, Besteiro S, Bhatia-Kissova I, Bi X, Biard-Piechaczyk M, Blum JS, Boise LH, Bonaldo P, Boone DL, Bornhauser BC, Bortoluci KR, Bossis I, Bost F, Bourquin JP, Boya P, Boyer-Guittaut M, Bozhkov PV, Brady NR, Brancolini C, Brech A, Brenman JE, Brennand A, Bresnick EH, Brest P, Bridges D, Bristol ML, Brookes PS, Brown EJ, Brumell JH, Brunetti-Pierri N, Brunk UT, Bulman DE, Bultman SJ, Bultynck G, Burbulla LF, Bursch W, Butchar JP, Buzgariu W, Bydlowski SP, Cadwell K, Cahova M, Cai D, Cai J, Cai Q, Calabretta B, Calvo-Garrido J, Camougrand N, Campanella M, Campos-Salinas J, Candi E, Cao L, Caplan AB, Carding SR, Cardoso SM, Carew JS, Carlin CR, Carmignac V, Carneiro LA, Carra S, Caruso RA, Casari G, Casas C, Castino R, Cebollero E, Cecconi F, Celli J, Chaachouay H, Chae HJ, Chai CY, Chan DC, Chan EY, Chang RC, Che CM, Chen CC, Chen GC, Chen GQ, Chen M, Chen Q, Chen SS, Chen W, Chen X, Chen YG, Chen Y, Chen YJ, Chen Z, Cheng A, Cheng CH, Cheng Y, Cheong H, Cheong JH, Cherry S, Chess-Williams R, Cheung ZH, Chevet E, Chiang HL, Chiarelli R, Chiba T, Chin LS, Chiou SH, Chisari FV, Cho CH, Cho DH, Choi AM, Choi D, Choi KS, Choi ME, Chouaib S, Choubey D, Choubey V, Chu CT, Chuang TH, Chueh SH, Chun T, Chwae YJ, Chye ML, Ciarcia R, Ciriolo MR,

Clague MJ, Clark RS, Clarke PG, Clarke R, Codogno P, Coller HA, Colombo MI, Comincini S, Condello M, Condorelli F, Cookson MR, Coombs GH, Coppens I, Corbalan R, Cossart P, Costelli P, Costes S, Coto-Montes A, Couve E, Coxon FP, Cregg JM, Crespo JL, Cronje MJ, Cuervo AM, Cullen JJ, Czaja MJ, D'Amelio M, Darfeuille-Michaud A, Davids LM, Davies FE, De Felici M, de Groot JF, de Haan CA, De Martino L, De Milito A, De Tata V, Debnath J, Degterev A, Dehay B, Delbridge LM, Demarchi F, Deng YZ, Dengjel J, Dent P, Denton D, Deretic V, Desai SD, Devenish RJ, Di Gioacchino M, Di Paolo G, Di Pietro C, Diaz-Araya G, Diaz-Laviada I, Diaz-Meco MT, Diaz-Nido J, Dikic I, Dinesh-Kumar SP, Ding WX, Distelhorst CW, Diwan A, Djavaheri-Mergny M, Dokudovskaya S, Dong Z, Dorsey FC, Dosenko V, Dowling JJ, Doxsey S, Dreux M, Drew ME, Duan Q, Duchosal MA, Duff K, Dugail I, Durbeej M, Duszenko M, Edelstein CL, Edinger AL, Egea G, Eichinger L, Eissa NT, Ekmekcioglu S, El-Deiry WS, Elazar Z, Elgendy M, Ellerby LM, Eng KE, Engelbrecht AM, Engelender S, Erenpreisa J, Escalante R, Esclatine A, Eskelinen EL, Espert L, Espina V, Fan H, Fan J, Fan QW, Fan Z, Fang S, Fang Y, Fanto M, Fanzani A, Farkas T, Farre JC, Faure M, Fechheimer M, Feng CG, Feng J, Feng Q, Feng Y, Fesus L, Feuer R, Figueiredo-Pereira ME, Fimia GM, Fingar DC, Finkbeiner S, Finkel T, Finley KD, Fiorito F, Fisher EA, Fisher PB, Flajolet M, Florez-McClure ML, Florio S, Fon EA, Fornai F, Fortunato F, Fotedar R, Fowler DH, Fox HS, Franco R, Frankel LB, Fransen M, Fuentes JM, Fueyo J, Fujii J, Fujisaki K, Fujita E, Fukuda M, Furukawa RH, Gaestel M, Gailly P, Gajewska M, Galliot B, Galy V, Ganesh S, Ganetzky B, Ganley IG, Gao FB, Gao GF, Gao J, Garcia L, Garcia-Manero G, Garcia-Marcos M, Garmyn M, Gartel AL, Gatti E, Gautel M, Gawriluk TR, Gegg ME, Geng J, Germain M, Gestwicki JE, Gewirtz DA, Ghavami S, Ghosh P, Giammarioli AM, Giatromanolaki AN, Gibson SB, Gilkerson RW, Ginger ML, Ginsberg HN, Golab J, Goligorsky MS, Golstein P, Gomez-Manzano C, Goncu E, Gongora C, Gonzalez CD, Gonzalez R, Gonzalez-Estevez C, Gonzalez-Polo RA, Gonzalez-Rey E, Gorbunov N, Part 2:, Gorski S, Goruppi S, Gottlieb RA, Gozuacik D, Granato GE, Grant GD, Green KN, Gregorc A, Gros F, Grose C, Grunt TW, Gual P, Guan JL, Guan KL, Guichard SM, Gukovskaya AS, Gukovsky I, Gunst J, Gustafsson AB, Halayko AJ, Hale AN, Halonen SK, Hamasaki M, Han F, Han T, Hancock MK, Hansen M, Harada H, Harada M, Hardt SE, Harper JW, Harris AL, Harris J, Harris SD, Hashimoto M, Haspel JA, Hayashi S, Hazelhurst LA, He C, He YW, Hebert MJ, Heidenreich KA, Helfrich MH, Helgason GV, Henske EP, Herman B, Herman PK, Hetz C, Hilfiker S, Hill JA, Hocking LJ, Hofman P, Hofmann TG, Hohfeld J, Holyoake TL, Hong MH, Hood DA, Hotamisligil GS, Houwerzijl EJ, Hoyer-Hansen M, Hu B, Hu CA, Hu HM, Hua Y, Huang C, Huang J, Huang S, Huang WP, Huber TB, Huh WK, Hung TH, Hupp TR, Hur GM, Hurley JB, Hussain SN, Hussey PJ, Hwang JJ, Hwang S, Ichihara A, Ilkhanizadeh S, Inoki K, Into T, Iovane V, Iovanna JL, Ip NY, Isaka Y, Ishida H, Isidoro C, Isobe K, Iwasaki A, Izquierdo M, Izumi Y, Jaakkola PM, Jaattela M, Jackson GR, Jackson WT, Janji B, Jendrach M, Jeon JH, Jeung EB, Jiang H, Jiang JX, Jiang M, Jiang Q, Jiang X, Jimenez A, Jin M, Jin S, Joe CO, Johansen T, Johnson DE, Johnson GV, Jones NL, Joseph B, Joseph SK, Joubert AM, Juhasz G, Juillerat-Jeanneret L, Jung CH, Jung YK, Kaarniranta K, Kaasik A, Kabuta T, Kadowaki M, Kagedal K, Kamada Y, Kaminskyy VO, Kampinga HH, Kanamori H, Kang C, Kang KB, Kang KI, Kang R, Kang YA, Kanki T, Kanneganti TD, Kanno H, Kanthasamy AG, Kanthasamy A, Karantza V, Kaushal GP, Kaushik S, Kawazoe Y, Ke PY, Kehrl JH, Kelekar A, Kerkhoff C, Kessel DH, Khalil H, Kiel JA, Kiger AA, Kihara A, Kim DR, Kim DH, Kim EK, Kim HR, Kim JS, Kim JH, Kim JC, Kim JK, Kim PK, Kim SW, Kim YS, Kim Y, Kimchi A, Kimmelman AC, King JS, Kinsella TJ, Kirkin V, Kirshenbaum LA, Kitamoto K, Kitazato K, Klein L, Klimecki WT, Klucken J, Knecht E, Ko BC, Koch JC, Koga H, Koh JY, Koh YH, Koike M, Komatsu M, Kominami E, Kong HJ, Kong WJ, Korolchuk VI, Kotake Y, Koukourakis MI, Kouri Flores JB, Kovacs AL, Kraft C, Krainc D, Kramer H, Kretz-Remy C, Krichevsky AM, Kroemer G, Kruger R, Krut O, Ktistakis NT, Kuan CY, Kucharczyk R, Kumar A, Kumar R, Kumar S, Kundu M, Kung HJ, Kurz T, Kwon HJ, La Spada AR, Lafont F, Lamark T, Landry J, Lane JD, Lapaquette P, Laporte JF, Laszlo L, Lavandero S, Lavoie JN, Layfield R, Lazo PA, Le W, Le Cam L, Ledbetter DJ, Lee AJ, Lee BW, Lee GM, Lee J, Lee JH, Lee M, Lee MS, Lee SH, Leeuwenburgh C, Legembre P, Legouis R, Lehmann M, Lei HY, Lei QY, Leib DA, Leiro J, Lemasters JJ, Lemoine A, Lesniak MS, Lev D, Levenson VV, Levine B, Levy E, Li F, Li JL, Li L, Li S, Li W, Li XJ, Li YB, Li YP, Liang C, Liang Q, Liao YF, Liberski PP, Lieberman A, Lim HJ, Lim KL, Lim K, Lin CF, Lin FC, Lin J, Lin JD, Lin K, Lin WW, Lin WC, Lin YL, Linden R, Lingor P, Lippincott-Schwartz J, Lisanti MP, Liton PB, Liu B, Liu CF, Liu K, Liu L, Liu QA, Liu W, Liu YC, Liu Y, Lockshin RA, Lok CN, Lonial S, Loos B, Lopez-Berestein G, Lopez-Otin C, Lossi L, Lotze MT, Low P, Lu B, Lu Z, Luciano F, Lukacs NW, Lund AH, Lynch-Day MA, Ma Y, Macian F, MacKeigan JP, Macleod KF, Madeo F, Maiuri L, Maiuri MC, Malagoli D, Malicdan MC, Malorni W, Man N, Mandelkow EM, Manon S, Manov I, Mao K, Mao X, Mao Z, Marambaud P, Marazziti D, Marcel YL, Marchbank K, Marchetti P, Marciniak SJ, Marcondes M, Mardi M, Marfe G, Marino G, Markaki M, Marten MR, Martin SJ, Martinand-Mari C, Martinet W, Martinez-Vicente M, Masini M, Matarrese P, Matsuo S, Matteoni R, Mayer A, Mazure NM, McConkey DJ, McConnell MJ, McDermott C, McDonald C, McInerney GM, McKenna SL, McLaughlin B, McLean PJ, McMaster CR, McQuibban GA, Meijer AJ, Meisler MH, Melendez A, Melia TJ, Melino G, Mena MA, Menendez JA, Menna-Barreto RF, Menon MB, Menzies FM, Mercer CA, Merighi A, Merry DE, Meschini S, Meyer CG, Meyer TF, Miao CY, Miao JY, Michels PA, Michiels C, Mijaljica D, Milojkovic A, Minucci S, Miracco C, Miranti CK, Mitroulis I, Miyazawa K, Mizushima N, Mograbi B, Mohseni S, Molero X, Mollereau B, Mollinedo F, Momoi T, Monastyrska I, Monick MM, Monteiro MJ, Moore MN, Mora R, Moreau K, Moreira PI, Moriyasu Y, Moscat J, Mostowy S, Mottram JC, Motyl T, Moussa CE, Muller S, Munger K, Munz C, Murphy LO, Murphy ME, Musaro A, Part 3:, Mysorekar I, Nagata E, Nagata K, Nahimana A, Nair U, Nakagawa T, Nakahira K, Nakano H, Nakatogawa H, Nanjundan M, Naqvi NI, Narendra DP, Narita M, Navarro M, Nawrocki ST, Nazarko TY, Nemchenko A, Netea MG, Neufeld TP, Ney PA, Nezis IP, Nguyen HP, Nie D, Nishino I, Nislow C, Nixon RA, Noda T, Noegel AA, Nogalska A, Noguchi S, Notterpek L, Novak I, Nozaki T, Nukina N, Nurnberger T, Nyfeler B, Obara K, Oberley TD, Oddo S, Ogawa M, Ohashi T, Okamoto K, Oleinick NL, Oliver FJ, Olsen LJ, Olsson S, Opota O, Osborne TF, Ostrander GK, Otsu K, Ou JH, Ouimet M, Overholtzer M, Ozpolat B, Paganetti P, Pagnini U, Pallet N, Palmer GE, Palumbo C, Pan T, Panaretakis T, Pandey UB, Papackova Z, Papassideri I, Paris I, Park J, Park OK, Parys JB, Parzych KR, Patschan S, Patterson C, Pattingre S, Pawelek JM, Peng J, Perlmutter DH, Perrotta I, Perry G, Pervaiz S, Peter M, Peters GJ, Petersen M, Petrovski G, Phang JM, Piacentini M, Pierre P, Pierrefite-Carle V, Pierron G, Pinkas-Kramarski R, Piras A, Piri N, Platanias LC, Poggeler S, Poirot M, Poletti A, Pous C, Pozuelo-Rubio M, Praetorius-Ibba M, Prasad A, Prescott M, Priault M, Produit-Zengaffinen N, Progulske-Fox A, Proikas-Cezanne T, Przedborski S, Przyklenk K, Puertollano R, Puyal J, Qian SB, Qin L, Qin ZH, Quaggin SE, Raben N, Rabinowich H, Rabkin SW, Rahman I, Rami A, Ramm G, Randall G, Randow F, Rao VA, Rathmell JC, Ravikumar B, Ray SK, Reed BH, Reed JC, Reggiori F, Regnier-Vigouroux A, Reichert AS, Reiners JJJr, Reiter RJ, Ren J, Revuelta JL, Rhodes CJ, Ritis K, Rizzo E, Robbins J, Roberge M, Roca H, Roccheri MC, Rocchi S, Rodemann HP, Rodriguez de Cordoba S, Rohrer B, Roninson IB, Rosen K, Rost-Roszkowska MM, Rouis M, Rouschop KM, Rovetta F, Rubin BP, Rubinsztein DC, Ruckdeschel K, Rucker EB3rd, Rudich A, Rudolf E, Ruiz-Opazo N, Russo R, Rusten TE, Ryan KM, Ryter SW, Sabatini DM, Sadoshima J, Saha T, Saitoh T, Sakagami H, Sakai Y, Salekdeh GH, Salomoni P, Salvaterra PM, Salvesen G, Salvioli R, Sanchez AM, Sanchez-Alcazar JA, Sanchez-Prieto R, Sandri M, Sankar U, Sansanwal P, Santambrogio L, Saran S, Sarkar S, Sarwal M, Sasakawa C, Sasnauskiene A, Sass M, Sato K, Sato M, Schapira AH, Scharl M, Schatzl HM, Scheper W, Schiaffino S, Schneider C, Schneider ME, Schneider-Stock R, Schoenlein PV, Schorderet DF, Schuller C, Schwartz GK, Scorrano L, Sealy L, Seglen PO, Segura-Aguilar J, Seiliez I, Seleverstov O, Sell C, Seo JB, Separovic D, Setaluri V, Setoguchi T, Settembre C, Shacka JJ, Shanmugam M, Shapiro IM, Shaulian E, Shaw RJ, Shelhamer JH, Shen HM, Shen WC, Sheng ZH, Shi Y, Shibuya K, Shidoji Y, Shieh JJ, Shih CM, Shimada Y, Shimizu S, Shintani T, Shirihai OS, Shore GC, Sibirny AA, Sidhu SB, Sikorska B, Silva-Zacarin EC, Simmons A, Simon AK, Simon HU, Simone C, Simonsen A, Sinclair DA, Singh R, Sinha D, Sinicrope FA, Sirko A, Siu PM, Sivridis E, Skop V, Skulachev VP, Slack RS, Smaili SS, Smith DR, Soengas MS, Soldati T, Song X, Sood AK, Soong TW, Sotgia F, Spector SA, Spies CD, Springer W, Srinivasula SM, Stefanis L, Steffan JS, Stendel R, Stenmark H, Stephanou A, Stern ST, Sternberg C, Stork B, Stralfors P, Subauste CS, Sui X, Sulzer D, Sun J, Sun SY, Sun ZJ, Sung JJ, Suzuki K, Suzuki T, Swanson MS, Swanton C, Sweeney ST, Sy LK, Szabadkai G, Tabas I, Taegtmeyer H, Tafani M, Takacs-Vellai K, Takano Y, Takegawa K, Takemura G, Takeshita F, Talbot NJ, Tan KS, Tanaka K, Tang D, Tanida I,

Tannous BA, Tavernarakis N, Taylor GS, Taylor GA, Taylor JP, Terada LS, Terman A, Tettamanti G, Thevissen K, Thompson CB, Thorburn A, Thumm M, Tian F, Tian Y, Tocchini-Valentini G, Tolkovsky AM, Tomino Y, Tonges L, Tooze SA, Tournier C, Tower J, Towns R, Trajkovic V, Travassos LH, Tsai TF, Tschan MP, Tsubata T, Tsung A, Turk B, Turner LS, Tyagi SC, Uchiyama Y, Ueno T, Umekawa M, Umemiya-Shirafuji R, Unni VK, Vaccaro MI, Valente EM, Van den Berghe G, van der Klei IJ, van Doorn W, van Dyk LF, van Egmond M, van Grunsven LA, Vandenabeele P, Vandenberghe WP, Vanhorebeek I, Vaquero EC, Velasco G, Vellai T, Vicencio JM, Vierstra RD, Vila M, Vindis C, Viola G, Viscomi MT, Voitsekhovskaja OV, von Haefen C, Votruba M, Wada K, Wade-Martins R, Walker CL, Walsh CM, Walter J, Wan XB, Wang A, Wang C, Wang D, Wang F, Wang G, Wang H, Wang HG, Wang HD, Wang J, Wang K, Wang M, Wang RC, Wang X, Wang YJ, Wang Y, Wang Z, Wang ZC, Wansink DG, Ward DM, Part 4:, Watada H, Waters SL, Webster P, Wei L, Weihl CC, Weiss WA, Welford SM, Wen LP, Whitehouse CA, Whitton JL, Whitworth AJ, Wileman T, Wiley JW, Wilkinson S, Willbold D, Williams RL, Williamson PR, Wouters BG, Wu C, Wu DC, Wu WK, Wyttenbach A, Xavier RJ, Xi Z, Xia P, Xiao G, Xie Z, Xu DZ, Xu J, Xu L, Xu X, Yamamoto A, Yamashina S, Yamashita M, Yan X, Yanagida M, Yang DS, Yang E, Yang JM, Yang SY, Yang W, Yang WY, Yang Z, Yao MC, Yao TP, Yeganeh B, Yen WL, Yin JJ, Yin XM, Yoo OJ, Yoon G, Yoon SY, Yorimitsu T, Yoshikawa Y, Yoshimori T, Yoshimoto K, You HJ, Youle RJ, Younes A, Yu L, Yu SW, Yu WH, Yuan ZM, Yue Z, Yun CH, Yuzaki M, Zabirnyk O, Silva-Zacarin E, Zacks D, Zacksenhaus E, Zaffaroni N, Zakeri Z, Zeh HJ3rd, Zeitlin SO, Zhang H, Zhang HL, Zhang J, Zhang JP, Zhang L, Zhang MY, Zhang XD, Zhao M, Zhao YF, Zhao Y, Zhao ZJ, Zheng X, Zhivotovsky B, Zhong Q, Zhou CZ, Zhu C, Zhu WG, Zhu XF, Zhu X, Zhu Y, Zoladek T, Zong WX, Zorzano A, Zschocke J, Zuckerbraun B. 2012. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy 8:445–544.

Korolchuk VI, Mansilla A, Menzies FM, Rubinsztein DC. 2009. Autophagy inhibition compromises degradation of ubiquitin-proteasome pathway substrates. Mol Cell 33:517–527.

Korolchuk VI, Menzies FM, Rubinsztein DC. 2010. Mechanisms of cross-talk between the ubiquitin-proteasome and autophagy-lysosome systems. FEBS Lett 584:1393–1398.

Laplante M, Sabatini DM. 2013. Regulation of mTORC1 and its impact on gene expression at a glance. J Cell Sci 126:1713–1719.

Li YP, Chen W, Liang Y, Li E, Stashenko P. 1999. Atp6i-deficient mice exhibit severe osteopetrosis due to loss of osteoclast-mediated extracellular acidification. Nat Genet 23:447–451.

Linares JF, Duran A, Yajima T, Pasparakis M, Moscat J, Diaz-Meco MT. 2013. K63 polyubiquitination and activation of mTOR by the p62-TRAF6 complex in nutrient-activated cells. Mol Cell 51:283–296.

Manolson MF, Yu H, Chen W, Yao Y, Li K, Lees RL, Heersche JN. 2003. The a3 isoform of the 100-kDa V-ATPase subunit is highly but differentially expressed in large (>or=10 nuclei) and small (<or= nuclei) osteoclasts. J Biol Chem 278:49271–49278.

Mao JH, Kim IJ, Wu D, Climent J, Kang HC, DelRosario R, Balmain A. 2008. FBXW7 targets mTOR for degradation and cooperates with PTEN in tumor suppression. Science 321:1499–1502.

Maxson ME, Grinstein S. 2014. The vacuolar-type H(+)-ATPase at a glance - more than a proton pump. J Cell Sci 127:4987–4993.

Mizushima N, Komatsu M. 2011. Autophagy: Renovation of cells and tissues. Cell 147:728–741.

Ochotny N, Flenniken AM, Owen C, Voronov I, Zirngibl RA, Osborne LR, Henderson JE, Adamson SL, Rossant J, Manolson MF, Aubin JE. 2011. The V-ATPase a3 subunit mutation R740S is dominant negative and results in osteopetrosis in mice. J Bone Miner Res 26:1484–1493.

Ochotny N, Voronov I, Owen C, Aubin JE, Manolson MF. 2013. The R740S mutation in the V-ATPase a3 subunit results in osteoclast apoptosis and defective early-stage autophagy. J Cell Biochem 114:2823–2833.

Park D, Lee MN, Jeong H, Koh A, Yang YR, Suh PG, Ryu SH. 2014. Parkin ubiquitinates mTOR to regulate mTORC1 activity under mitochondrial stress. Cell Signal 26:2122–2130.

Petherick KJ, Williams AC, Lane JD, Ordonez-Moran P, Huelsken J, Collard TJ, Smartt HJ, Batson J, Malik K, Paraskeva C, Greenhough A. 2013. Autolysosomal beta-catenin degradation regulates Wnt-autophagy-p62 crosstalk. Embo J 32:1903–1916.

Sancak Y, Bar-Peled L, Zoncu R, Markhard AL, Nada S, Sabatini DM. 2010. Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell 141:290–303.

Settembre C, Fraldi A, Medina DL, Ballabio A. 2013. Signals from the lysosome: A control centre for cellular clearance and energy metabolism. Nat Rev Mol Cell Biol 14:283–296.

Takayanagi H. 2007. The role of NFAT in osteoclast formation. Ann N Y Acad Sci 1116:227–237.

Toyomura T, Murata Y, Yamamoto A, Oka T, Sun-Wada GH, Wada Y, Futai M. 2003. From lysosomes to the plasma membrane: Localization of vacuolar-type H+ -ATPase with the a3 isoform during osteoclast differentiation. J Biol Chem 278:22023–22030.

Toyomura T, Oka T, Yamaguchi C, Wada Y, Futai M. 2000. Three subunit a isoforms of mouse vacuolar H(+)-ATPase. Preferential expression of the a3 isoform during osteoclast differentiation. J Biol Chem 275:8760–8765.

Voronov I, Ochotny N, Jaumouille V, Owen C, Manolson MF, Aubin JE. 2013. The R740S mutation in the V-ATPase a3 subunit increases lysosomal pH, impairs NFATc1 translocation, and decreases in vitro osteoclastogenesis. J Bone Miner Res 28:108–118.

Wang XJ, Yu J, Wong SH, Cheng AS, Chan FK, Ng SS, Cho CH, Sung JJ, Wu WK. 2013. A novel crosstalk between two major protein degradation systems: Regulation of proteasomal activity by autophagy. Autophagy 9(10):1500–1508.

Yu S, Shen G, Khor TO, Kim JH, Kong AN. 2008. Curcumin inhibits Akt/mammalian target of rapamycin signaling through protein phosphatase-dependent mechanism. Mol Cancer Ther 7:2609–2620.

Zoncu R, Bar-Peled L, Efeyan A, Wang S, Sancak Y, Sabatini DM. 2011. MTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H(+)-ATPase. Science 334:678–683.