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GSK-3-TSC axis governs lysosomal acidification through autophagy and endocytic pathways

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ABSTRACT

Impaired lysosomal activity, which results in defective protein processing, waste accumulation, and protein aggregation, is implicated in a number of disease pathologies. Acidification of lysosomes is a crucial process required for lysosome function. Previously we showed that inhibition of glycogen synthase kinase-3 (GSK-3) enhanced lysosomal acidification in both normal and pathological conditions. However, how GSK-3 integrates into the lysosome networking is unknown. Here we show that inhibition of mTORC1 and increased autophagic activity are downstream to GSK-3 inhibition and contribute to lysosomal acidification. Strikingly, lysosomal acidification is also restored by GSK-3 inhibition in the absence of functional autophagy, and, independently of mTORC1. This is facilitated by increased endocytic traffic: We show that GSK-3 inhibition enhanced material internalization, increased recruitment of active Rab5 into endosomes, and increased Rab7/RILP clustering into lysosomes, all processes required for late endosome maturation. Consistently, in cells defective in endocytic traffic caused by either constitutively active Rab5, or, deletion of the Niemann-Pick C1 protein, GSK-3 inhibition could not restore lysosomal acidification. Finally we found that the tuberous sclerosis complex, TSC, is required for lysosomal acidification and is activated by GSK-3 inhibition. Thus, the GSK-3/TSC axis regulates lysosomal acidification via both the autophagic and endocytic pathways. Our study provides new insights into the therapeutic potential of GSK-3 inhibitors in treating pathological conditions associated with impaired cellular clearance.

1. Introduction

Efficient clearance of cellular waste is vital for maintaining normal cell, tissue, and organism homeostasis [1–6]. Impairments in protein turnover cause numerous human diseases including metabolic disorders, cancer, and neurodegeneration [1–5,7]. Lysosomes serve as the end point of autophagy and endocytosis, the major degradative pathways through which cellular material is recruited and destined for degradation [8–11]. Hence, lysosomes are critical hubs that coordinate protein turnover and that integrate sensing mechanisms for growth factors and nutrient signaling. The lysosome is an acidic organelle, and the low pH is maintained by the v-ATPase proton pump, a large multimeric protein complex [12,13]. Acidification is crucial for optimal function of lysosomes [8,9,14]. Reduced lysosomal acidification has been reported in aging subjects and in patients with pathological disorders including Alzheimer's, Parkinson's, and Huntington diseases as well as metabolic disorders and cancer, making lysosomes a potential therapeutic target [1,2,7,8,15–17]. However, an understanding of how lysosomal function is manipulated in different cellular states remains elusive.

Studies show that lysosomes undergo sophisticated regulation controlled by multiple components. For example, localization of the mammalian target of rapamycin complex 1 (mTORC1), a master regulator of cellular growth and metabolism [18–21], to the lysosome surface upon nutrient stimulation results in mTORC1 activation by the Rheb GTPase [22,23]. Lysosome biogenesis that is triggered by suppression of mTORC1 and activation of the transcription factor EB (TFEB) results in upregulation of v-ATPase [25,26] contributing to lysosomal acidification. Alterations in lysosome positioning also influence the cellular distribution and activation state of mTORC1 [24]. Autophagy, a main pathway for sequestration and targeting of cytoplasmic components to the lytic compartment [27–30], is another

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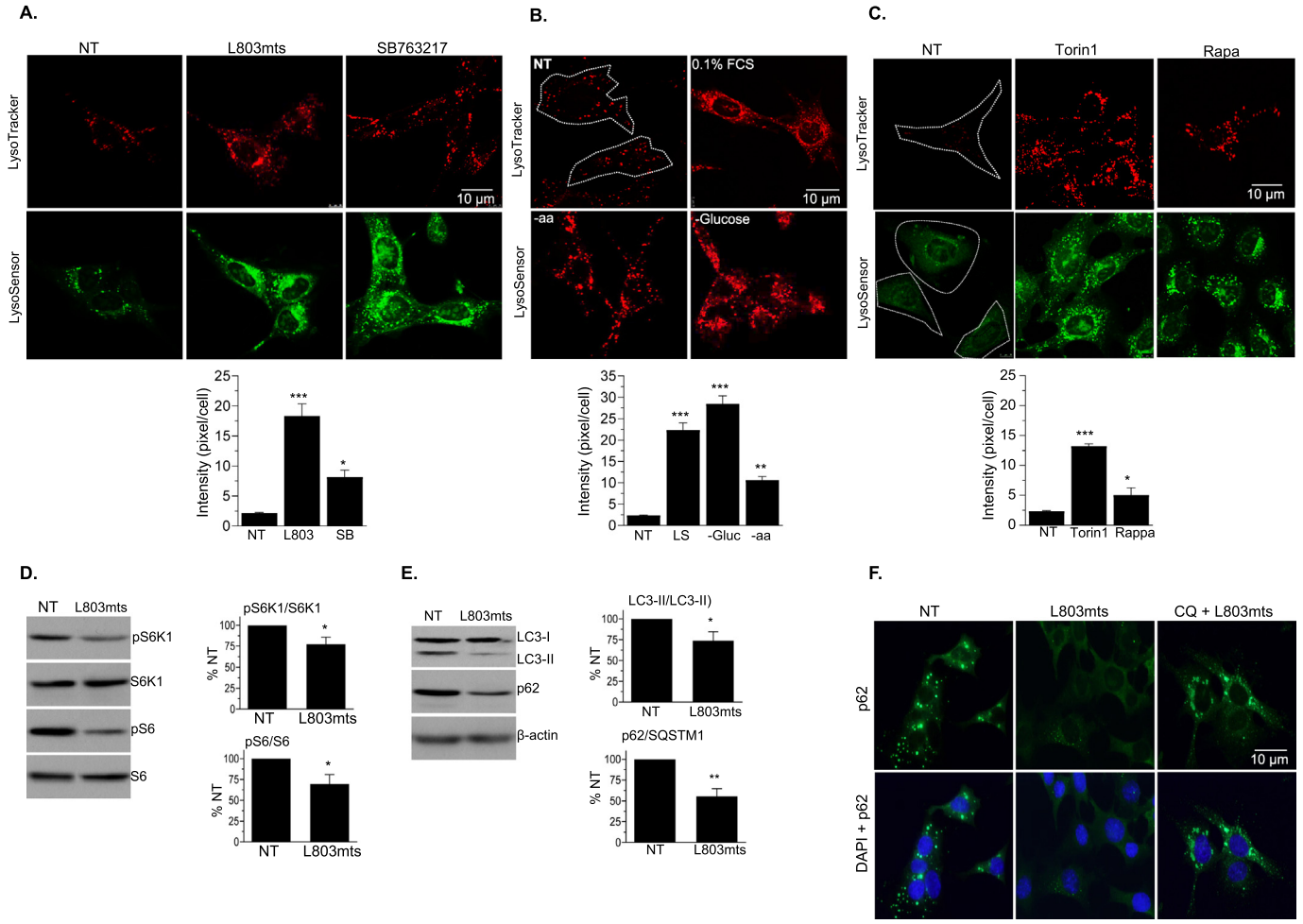

Fig. 1. mTORC1 and autophagy are downstream to GSK-3 and jointly contribute to lysosomal acidification. A. MEF PS1/2$^{-/-}$ cells were treated with L803mts (40 mM) or SB763217 5 (mM) for 2 h. The cells were stained with LysoTracker Red or LysoSensor Green and imaged by confocal microscopy. B. MEF PS1/2$^{-/-}$ cells were incubated with 0.1% FCS, no amino acids (−aa), or no glucose for 4 h, stained with LysoTracker Red or LysoSensor Green, and imaged by confocal microscopy. C. MEF PS1/2$^{-/-}$ Cells were treated with Torin1 (200 nM) or rapamycin (Rapa) (50 nM) for 2 h. Cells were stained with LysoTracker Red or LysoSensor Green and imaged by confocal microscopy. Cells with low fluorescent signal are indicated by dashed lines. D. Phosphorylation of mTORC1 targets, S6K-1 and S6, was detected by immunoblot analysis in MEF PS1/2$^{-/-}$ cells that were treated with L803mts as described. E. Autophagic markers LC3II and p62/SQSTM1 (p62) detected in MEF PS1/2$^{-/-}$ cells treated with L803mts as described. For panels D and E, means ± SEM of densitometry analysis are shown to the right and presented as % of non-treated. * $p < .05$, ** $p < .01$ treated vs. non-treated by student's *t-test*, $n = 3$–5. F. MEF PS1/2$^{-/-}$ cells were treated with L803mts (40 mM) for 2 h with or without chloroquine (CQ) (30 μM). Cells were fixed and stained with anti p62/SQSTM1 antibody. Merged images with DAPI are shown at the lower panel. For panel B&C dashed line indicates cells with low fluorescent signal. For all panels, images shown are representative of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

potential player in lysosomal regulation. In response to stress and starvation, new autophagosomes are generated and fuse with lysosomes [27–30]. This fusion process appears to increase lysosomal acidification [31,32]. Activation of autophagy by other signaling components, such as the Abelson tyrosine kinase, also promotes lysosomal traffic [33]. Finally, late endosome maturation through the endocytic pathway facilitates vesicle acidification [11,34,35].

In a recent work we showed that glycogen kinase synthase-3 (GSK-3) is a negative regulator of lysosome function: Overexpression of GSK-3 impairs lysosomal acidification, whereas treatment with GSK-3 inhibitors significantly increases lysosomal acidification [36,37]. Moreover, GSK-3 inhibition results in recovery of lysosomal acidification in cells that were deficient in lysosomal acidification [36,37] and in animal models of Alzheimer's disease [36], a disease associated with poor lysosomal acidification [1,17,38,39]. GSK-3 is a key regulator of various cellular processes and has emerged as a prominent drug discovery target [40–43]. The role of GSK-3 in regulating lysosomes provides new insight into how therapeutic benefits are achieved with GSK-3 inhibitors in pathological conditions associated with impaired clearance activity.

In this study we deepened our investigation to uncover mechanisms linking GSK-3 with the lysosome networking. We show that GSK-3 inhibition enables lysosomal acidification through mTORC1/autophagy and the endocytic pathways. We further demonstrate that tuberous sclerosis complex (TSC) and Rab5 are key players in this process.

2. Results

2.1. mTORC1/autophagy are downstream to GSK-3 and contribute to lysosomal acidification

In previous work we showed that inhibition of GSK-3 restores lysosomal acidification in MEF cells lacking presenilin proteins [44]. Fig. 1A shows that treatment of the MEF PS1/2$^{-/-}$ cells with either of two GSK-3 inhibitors, L803mts, a selective peptide GSK-3 inhibitor developed in our laboratory [45], or SB763217, a small-molecule ATP competitive inhibitor [46], recovered impaired acidification of the lysosomal pool in the MEF PS1/2$^{-/-}$ cells. Experiments were performed

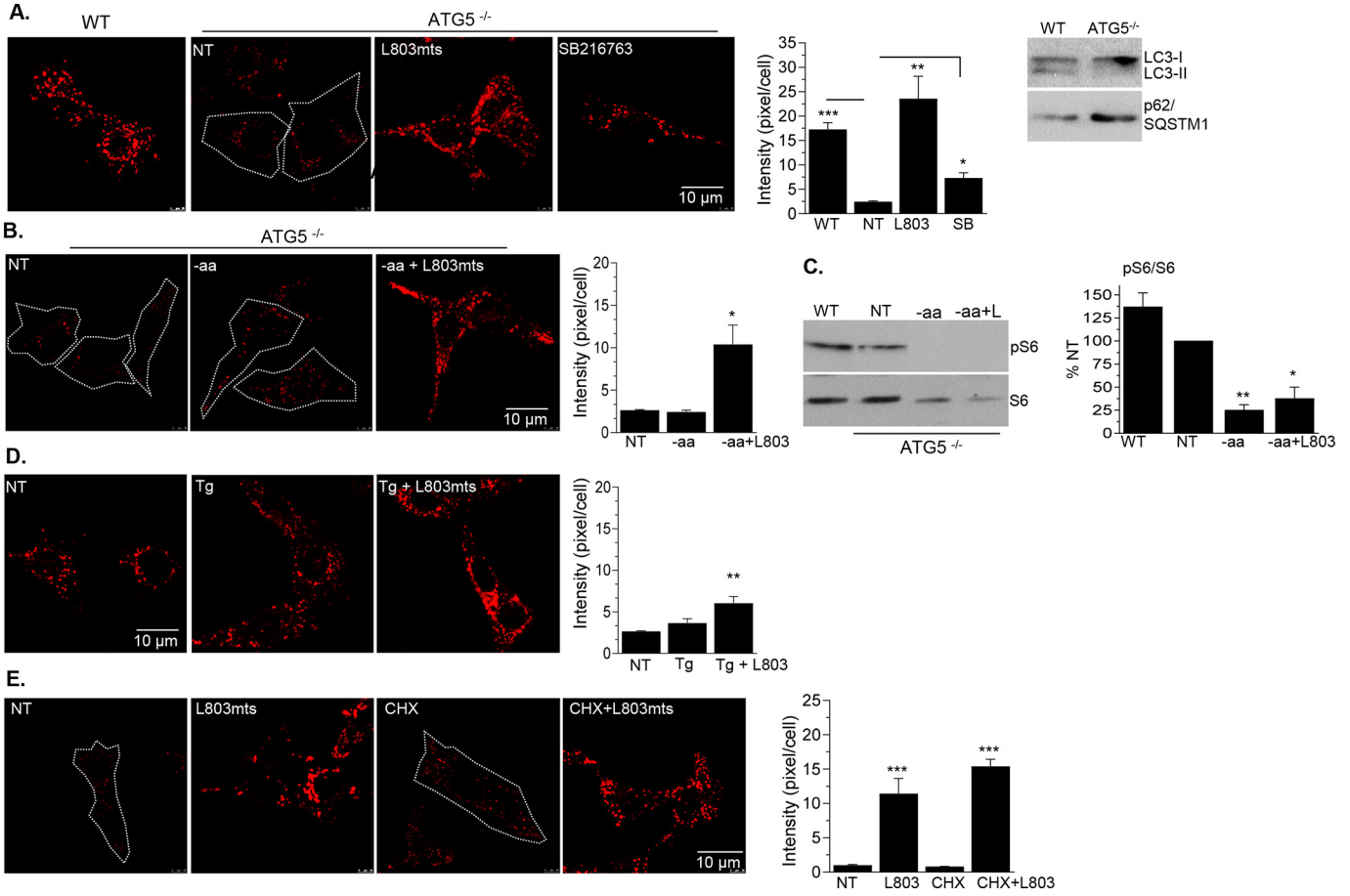

Fig. 2. GSK-3 inhibition induces lysosomal acidification independent of autophagy, or, mTORC1. A. MEF (WT) and ATG5$^{-/-}$ cells were treated with L803mts (40 mM) or SB763217 5 (mM) for 2 h. *Left panel:* Cells were stained with LysoTracker Red and imaged by confocal microscopy. Right panel: Extracts were immunoblotted for autophagy markers. B. Left: ATG5$^{-/-}$ cells were starved for amino acids (−aa) and treated or not with L803mts as described. Cells were stained with LysoTracker Red and imaged by confocal microscopy. Right: Phosphorylation of S6 was determined by immunoblot analysis in extracts prepared from starved ATG5$^{-/-}$ cells (−aa) and treated or not with L803mts (−aa+L). Means ± SEM based on densitometry analysis is shown. $^{**}p < .01$, treated vs. non-treated by Student's *t-test*, $n = 3$. a.u., arbitrary units C. MEF cells were treated with thapsigargine (Tg) (3 μM) with or without L803mts as described. Cells were stained with LysoTracker Red and imaged by confocal microscopy D. MEF cells were treated with cycloheximide (10 mM) with or without L803mts. Cells were stained with LysoTracker Red and imaged by confocal microscopy. For panels A, B, D. dashed line indicates cells with low fluorescent signal. NT indicates non-treated. Images shown are representative of 3 independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in live cells stained with LysoTracker Red, a dye that accumulates in acidic vesicles and in cells stained with LysoSensor Green, which confirmed lysosomal pH was approximately 4.5. We further found that starvation signals, such as low serum, depletion of amino acids, or lack of glucose, significantly increased lysosomal acidification in these cells (Fig. 1B). This indicated a likely involvement of mTORC1, as mTORC1 activity is suppressed by stress and starvation signals [18–21]. To verify this, we showed that treatment with mTORC1 inhibitors Torin1 and rapamycin enhanced lysosomal acidification (Fig. 1C). Further, treatment with the GSK-3 inhibitor L803mts reduced mTORC1 activity relative to levels in untreated cells as indicated by reduced phosphorylation levels of the mTORC1 target p70 ribosomal S6 kinase (S6K-1, Thr^{389}) and the S6K-1 target ribosomal S6 protein (S6, Ser$^{240/244}$) (Fig. 1D). Together, our data show that reduced lysosomal acidification in MEF PS1/2$^{-/-}$ cells was 'repaired' by starvation signals or by GSK-3 inhibition. These results support the lysosome adaptation paradigm [47] and also indicate that GSK-3-mediated lysosomal acidification is coupled with mTORC1 signaling.

Cells respond to starvation and stress signals via suppression of mTORC1, which in turn enhances autophagy Increased autophagy may trigger lysosomal acidification during the fusion process of autophagosomes with lysosomes [31,32]. Thus, we asked whether GSK-3 regulates autophagic activity. Two established autophagic markers were evaluated: light chain 3II (LC3II), which is used as an index for the number of autophagosomes [48], and p62/SQSTM1 (also called sequestosome 1), which is directly involved in delivery of ubiquitinated proteins into autophagosomes and which is itself degraded by autophagy [49]. Immunoblot analysis indicated that L803mts reduced levels of LC3II and the LC3II/LC3I ratio (Fig. 1E). Since levels of LC3II are dynamically altered [50], it is possible that autophagy was either enhanced or slowed due to defective lysosomal activity. We also found that levels of p62/SQSTM1 were reduced in response to L803mts (Fig. 1E). Collectively, these results indicated that inhibition of GSK-3 results in increased autophagic turnover. Further support for this conclusion was obtained by immunofluorescence analyses that probed for endogenous p62/SQSTM1 in the presence or absence of chloroquine (CQ), a lysomotrophic drug that neutralizes lysosomal pH. There were significantly fewer p62/SQSTM1 puncta in cells treated with L803mts and blocked with CQ than in cells treated only with CQ (Fig. 1F). Taken together, these data show that GSK-3 inhibition induces lysosomal acidification and is associated with suppressed mTORC1 and enhanced autophagic activity.

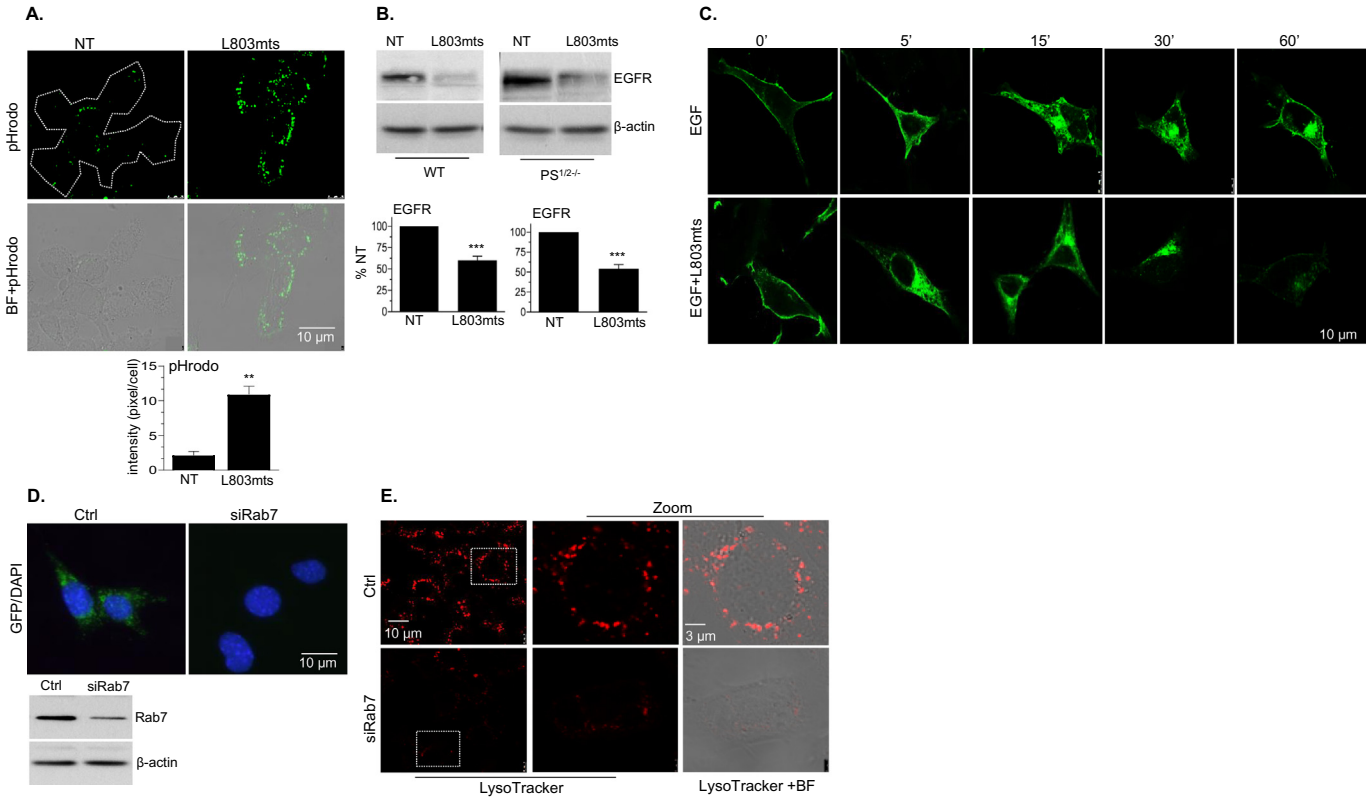

Fig. 3. GSK-3 regulates endocytosis. A. MEF cells were treated with L803mts (40 μM) for 2 h and then incubated with pHrodo green dextran (200 μg/ml) for 15 min. Images of live cells stained with pHrodo (upper panel) and merged signals with bright field (BF) (lower panel) is shown. Dashed line indicates cells with low fluorescent signal. Bar graph of quantitation of pHrodo signal is shown. **p < .01 treaded vs. non treated by Student's *t-test,* n = 3. B. Levels of EGFR were determined using immunoblot analysis in MEF (WT) or PS1/2$^{-/-}$ MEF cells that were treated with L803mts as described. Means ± SEM based on densitometry analysis is shown in lower panel and presented as % of NT. ***p < .001 treaded vs. non treated by Student's *t-test,* n = 3. C. MEF cells expressing GFP-EGFR were treated or not with 40 μM L803mts (40 μM) . GFP-EGFR signal was monitored after treatment with EGF (50 ng/ml) for indicated time points in PFA-fixed cells. D. Rab7 was silenced in MEF cells. Cells were fixed and immunostained with anti-Rab7 antibody. Scrambled RNA was used as a control (ctrl). Merged signals of Rab7 and DAPI are shown. Expression of Rab7 was determined by immunoblot analysis and shown at the lower panel. E. Cells described in panel D were stained with LysoTracker Red and imaged by con-focal microscopy. Magnified regions (marked boxes) are shown at the middle and right panels All panels show representative images from 3 independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. GSK-3-mediated lysosomal acidification is not exclusively dependent on autophagy or mTORC1

We then sought to determine whether or not the mTORC1/autophagy axis is the sole mechanism that controls GSK-3-mediated lysosomal acidification. For this, we used ATG5$^{-/-}$ MEF cells that lack autophagosomes and do not convert LC3I to LC3II [51]. As expected, ATG5$^{-/-}$ MEF cells lacked LC3II and showed abnormal accumulation of p62/SQSTM1 (Fig. 2A right). Lysosomal acidification is very low in these cells as compared to wild-type MEF cells (Fig. 2A left), indicating that autophagy is indeed an important player in lysosomal acidification. Unexpectedly, treatment with L803mts or with SB216763 was able to recover lysosomal acidification in the ATG5$^{-/-}$ cells (Fig. 2A left). In addition, whereas starvation failed to restore lysosomal acidification, L803mts treatment did acidify lysosomes under these conditions in which mTORC1 activity was suppressed (Fig. 2B right). These results indicate that GSK-3 inhibition results in acidification of lysosomes through a pathway that is not necessarily dependent on autophagy or mTORC1. Further support for this conclusion came from studies with thapsigargin, which specifically prevents membrane fusion of autophagosomes with lysosomes [26]. Treatment with thapsigargin did not prevent L803mts-induced acidification of lysosomes (Fig. 2C). Finally, we examined if elevation in lysosomal acidification reflected de novo lysosome biogenesis that 'escaped' the demand for autophagy. Thus, cells were treated with cycloheximide to block synthesis of new proteins. This, however, did not prevent the L803mts-mediated increase in lysosomal acidification (Fig. 2D). We conclude, therefore, that GSK-3 inhibition activates an alternative autophagy-independent pathway that results in acidification of lysosomes.

2.3. GSK-3 inhibition enhances flux through the endocytic pathway

As lysosomes are located at the crossroads of autophagy and the endocytic pathway [11,34,52] and their maturation is coordinated with endocytosis [53], it is possible that the endocytic pathway is the alternative autophagy-independent pathway that facilitates lysosomal acidification upon GSK-3 inhibition. To test this, we first determined whether GSK-3 inhibition upregulates endocytosis. MEF cells were treated with L803mts followed by incubation with pHrhodo green dextran. This reagent is pH sensitive and fluoresces upon entry into acidic vesicles. Addition of L803mts to cells increased the cellular accumulation of pHrhodo green dextran (Fig. 3A). Internalization of the epidermal growth factor receptor (EGFR), which is ultimately degraded in lysosomes [54–56], also serves as a marker for endocytosis. Steady-state levels of EGFR were reduced by L803mts treatment in both MEF and MEF PS1/2$^{-/-}$ cells relative to levels in untreated cells (Fig. 3B). EGFR internalization was also monitored in MEF cells expressing GFP-labeled EGFR followed by treatment with EGF ligand. Treatment with EGF initially increased the GFP-EGFR signal in the cytoplasm, and signal decayed within 60 min (Fig. 3C). Treatment with L803mts prior to addition of EGF accelerated the signal decay with decay by 30 min (Fig. 3C).

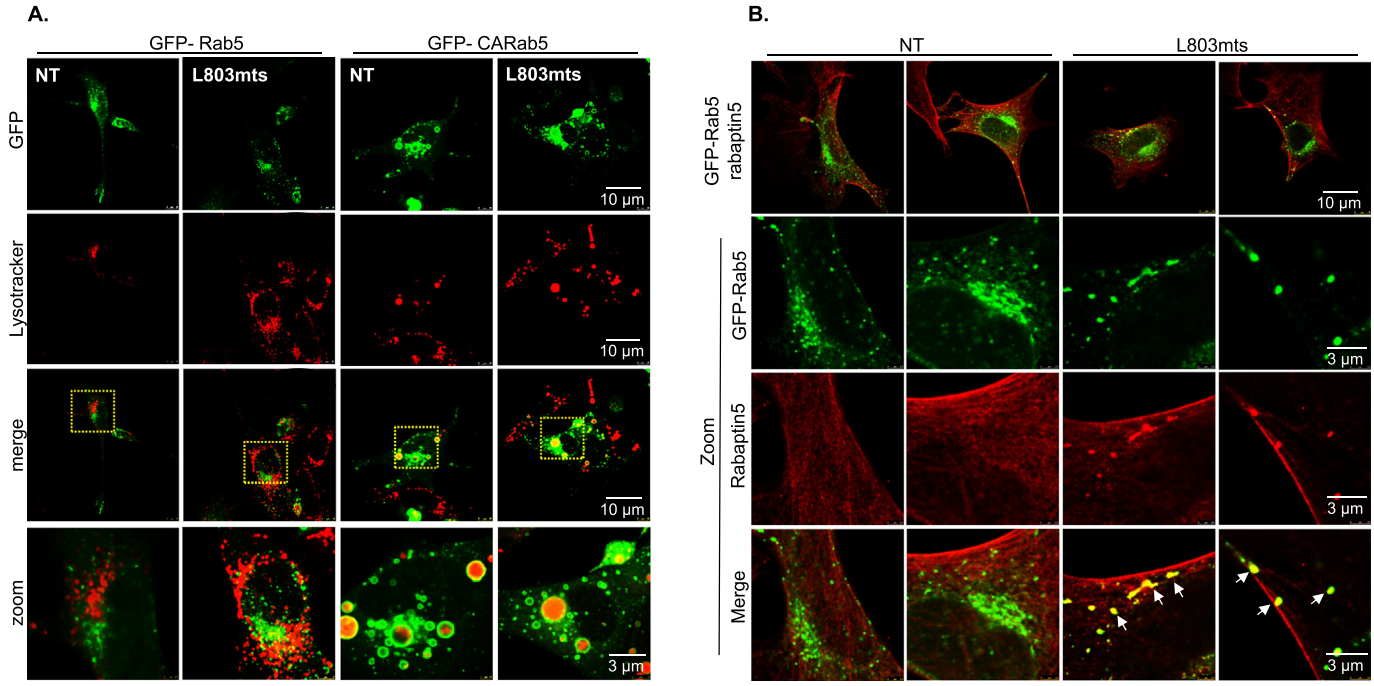

Fig. 4. GSK-3-mediated lysosomal acidification is dependent of Rab5. A. MEF cells expressing GFP-tagged Rab5 (GFP-Rab5) or constitutively active mutant Rab5 (GFP-CARab5) were treated with or without L803mts (40 μM) for 2 h. Cells were stained with LysoTracker Red and imaged by confocal microscopy. Magnified regions (marked in boxes) are shown in the lower panel. B. MEF cells expressing GFP-Rab5 were treated with or without L803mts as described. PFA-fixed cells were immunostained with anti-rabaptin-5 antibody. Upper panel shows merged signals of GFP-Rab5 (green) and rabaptin-5 (red). Magnified regions are shown in the lower panels. White arrows indicate co-localization of GFP-Rab5 and rabaptin-5 (lowest panel). Representative images of 3 independent experiments are shown for panels A and B. NT, non-treated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To further verify that endocytosis can induce lysosomal acidification, we silenced expression of the small GTPase Rab7, an integral component of the endosome/lysosome maturation [57–63], with a specific siRNA (Fig. 3D). Reduction in Rab7 effectively reduced lysosomal acidification (Fig. 3E). Taken together, our data suggest that effects on GSK-3-mediated endocytosis are responsible for lysosomal acidification induced by inhibition of GSK-3.

2.4. GSK-3-mediated effects on lysosomal acidification are dependent on Rab5 and endocytic traffic

The small GTPase Rab5 is a major player in directing endocytic traffic [53,61,63–68]. It exists in active (GTP-bound) or inactive (GDP-bound) states. Expression of the constitutively active Rab5 mutant Rab5Q^{79}L (CARab5) that is deficient in GTPase activity was shown to disrupt endosome maturation [57,69]. MEF cells were transfected with constructs for expression of GFP-tagged wild-type (WT) Rab5 or CARab5 and their impacts on the ability of L803mts to acidify lysosomes were measured. The expression of WT-Rab5 did not interfere with L803mts-induced lysosomal acidification (Fig. 4A). However, cells expressing CARab5 failed to acidify lysosomes in response to L803mts (Fig. 4A). The cells that express CARab5 exhibited enlarged endosomes, and the number of LysoTracker Red puncta was reduced and clustered in part near the expanded endosome structures (Fig. 4A). The enlarged endosomes are mixtures of early and late endosomes caused by impairment in Rab5 normal activity [53,57,70]. Treatment with L803mts did not 'correct' the appearance of the enlarged structures nor did it enhance the LysoTracker Red signal in CARab5-expressing cells (Fig. 4A).

We next examined whether Rab5 is regulated by GSK-3. The active GTP-bound Rab5 forms a complex with its effector rabaptin-5 [61,71]. Thus, formation of the Rab5 complex with rabaptin5 was determined in cells expressing GFP-Rab5 that were treated with L803mts by immunostaining with anti-rabaptin-5 antibody. Indeed, L803mts increased co-localization of GFP-Rab5 with rabaptin5, and the merged signal was specifically localized at large structures (Fig. 4B). These structures reflect high density localization of active Rab5 [57]. Thus, inhibition of GSK-3 increases the active pool of Rab5 as reflected by the complex formation with rabaptin5. This, in turn, accelerates endosome maturation and lysosomal acidification.

2.5. GSK-3 inhibition increases Rab7 transport to lysosomes

Rab5-Rab7 exchange promotes early to late endosome conversion, which is manifested by Rab7 transport to late endosomes and lysosomes [57–61,62,68,72,73]. If GSK-3 inhibition accelerates endocytic traffic, we expected to find more Rab7 at the lysosome surface after treatment of cells with L803mts. MEF cells were treated or not with L803mts and were co-immunostained with antibodies against Rab7 and the lysosome marker Lamp2; co-localization of Rab7 with Lamp2 was higher in cells treated with L803mts (Fig. 5A). The expression of the dominant negative mutant of Rab7, T222N, a mutant that cannot bind GTP, resulted in the formation of large vacuoles that were partially acidified (Fig. 5B). Treatment with L803mts partially rescued this phenomenon (Fig. 5B). This likely due to the upload of endogenous Rab7 into lysosomes in response to L803mts. The expression of the Rab7 effector RILP marks vacuoles that are not acidified (likely lysosomes) [58,59,74]. Treatment with L803mts resulted in vacuole acidification (Fig. 5C), further showing that recruitment of Rab7 into lysosomes is enhanced by inhibition of GSK-3. Taken together, our data show that GSK-3 inhibition promotes Rab7 trafficking into lysosomes and supports our notion that GSK-3 inhibition accelerates early to late endosome conversion.

2.6. Endocytic trafficking is required for GSK-3 mediated lysosome acidification

We speculated that the acidification observed in the MEF ATG5$^{-/-}$ cells treated with L803mts (Fig. 2) was likely enabled due the endocytic

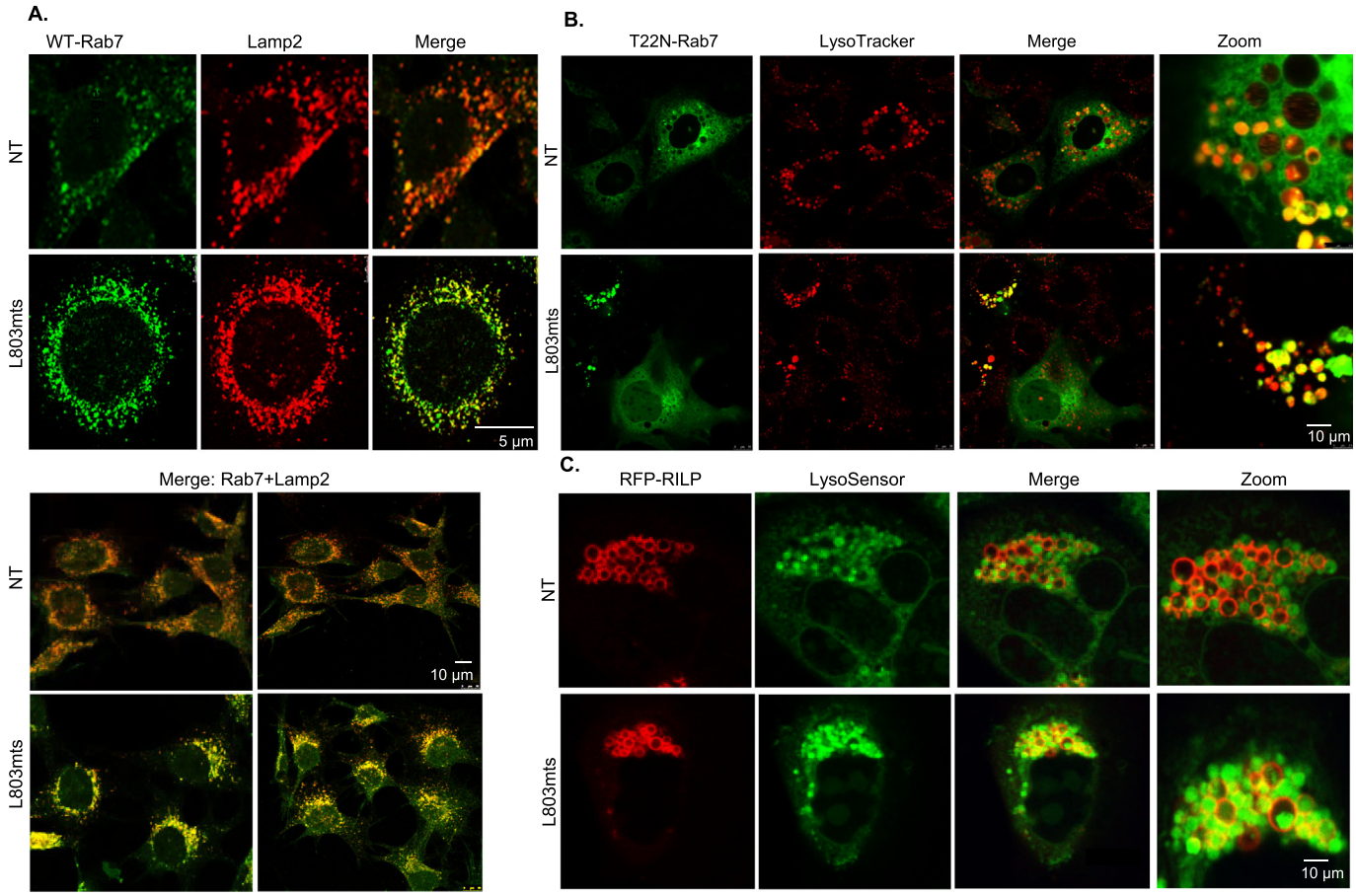

Fig. 5. GSK-3 inhibition promotes Rab7 clustering at the lysosomes. A. MEF cells were treated with or without L803mts (40 μM) for 2 h. The cells were PFA fixed and co-immunostained with anti-Rab7 and anti-Lamp2 antibodies. Upper panel shows staining in single cells, lower panel shows merged signals of Rab7 (green) and Lamp2 (red). B. MEF cells were transfected with GFP-T22N-Rab7 mutant. Cells were treated or not treated with L803mts as described and stained with LysoTracker Red. Live cells were imaged by confocal microscopy. C. MEF cells expressing RFP-RILP were treated or not with L803mts as described. Cells were stained with LysoSensor Green and imaged by confocal microscopy. Representative images from 3 independent experiments are shown. NT, non-treated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pathway. To confirm this, MEF ATG5$^{-/-}$ cells were transfected with CARab5 to disrupt endocytic trafficking and then treated with L803mts. Indeed, no increase in lysosomal acidification was observed in these cells as compared to cells expressing WT-Rab7 (Fig. 6A).

Nieman Pick type C1 (NPC1) disease is an inherited lysosomal storage disorder in which cholesterol accumulates in late endosomes due to disrupted endocytic trafficking [75,76]. The disease is caused by mutations in NPC1 protein [77,78]. Thus we used *NPC1*-null CHO cells to further test our hypothesis. We found that treatment with L803mts did not increase lysosomal acidification in the *NPC1*-null CHO cells (Fig. 6B). Treatment of *NPC1*-null CHO cells with CQ showed reduced LysoTracker Red signal relative to wild-type CHO cells, confirming that lysosomal pH could be altered in these cells (Fig. 6B). Finally, we treated cells with vinblastine that prevents vesicle fusion and endosome maturation. Treatment with L803mts could not restore reduction in lysosomal acidification induced by vinblastine (Fig. 6C). Taken together, intact endocytic traffic is a necessary pathway required for lysosomal acidification induced by GSK-3 inhibition.

2.7. Role of tuberous sclerosis complex in GSK-3-mediated lysosomal acidification

GSK-3 was shown to mediate mTORC1 activity via the TSC, a complex composed of TSC1 and TSC2 that is an upstream negative regulator of mTORC1 [79–82]. To further determine whether or not the GSK-3/TSC axis is crucial to lysosomal acidification, we used MEF cells lacking TSC1 and 2 (TSC1/2$^{-/-}$ cells) that have constitutively active mTORC1 [83]. Lysosomal acidification was lower in TSC1/2$^{-/-}$ cells than control cells; however, starvation or treatment with rapamycin recovered lysosomal acidification in the mutant cells (Fig. 7A). In contrast, treatment of TSC1/2$^{-/-}$ cells with L803mts did not re-acidify lysosomes, inhibit mTORC1 activity, or alter autophagic activity (Fig. 7A).

To further confirm that TSC is a downstream target of GSK-3 we evaluated TSC cellular localization after treatment with L803mts. Previous studies showed that translocation of TSC to lysosomes is observed upon mTORC1 inhibition [84,85]. Cells were treated with three different GSK-3 inhibitors, L803mts, SB216763, or lithium. Following treatment, PFA-fixed cells were co-immunostained with anti-TSC2 and anti-Lamp2 antibodies. The GSK-3 inhibitors increased co-localization of TSC2 and Lamp2 relative to untreated cells (Fig. 7B). In addition, we showed that the phosphorylated pool of active mTOR (pSer^{2448}) at the lysosome surface was reduced following treatment with L803mts (Fig. 7C). Taken together, our results indicate that GSK-3 is a negative regulator of TSC. Furthermore, the effects of GSK-3 on lysosomal acidification that are dependent on the mTORC1 pathway require functional TSC.

3. Discussion

In this study we investigated the molecular mechanisms underlying GSK-3 regulation of lysosome activity with a particular focus on

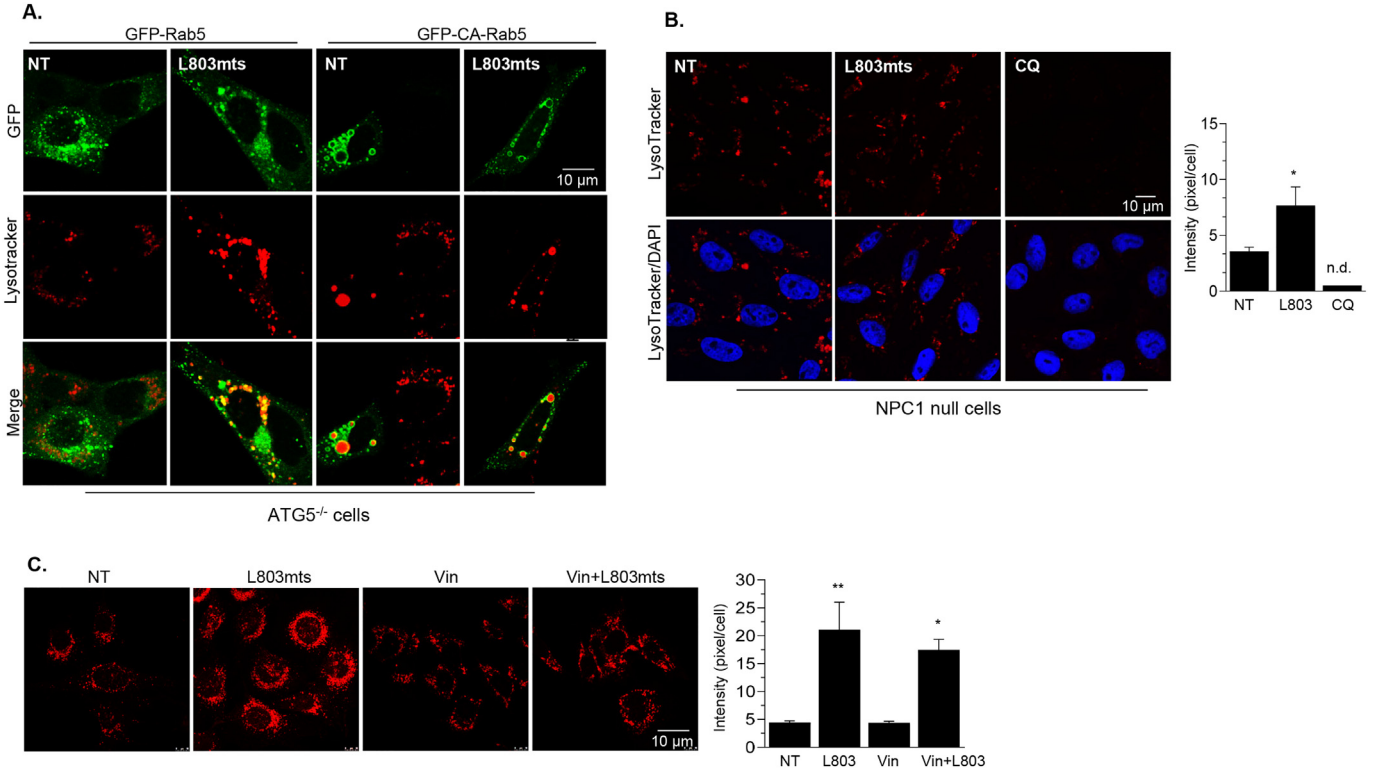

Fig. 6. Impaired endocytic traffic disables GSK-3 inhibition-induced lysosomal acidification A. ATG5$^{-/-}$ cells were transfected with GFP-Rab5 or GFP-CARab5 mutant constructs. Cells were treated with or without L803mts (40 μM) for 2 h. Cells were stained with LysoTracker Red and imaged by confocal microscopy. B. NPC1-null CHO cells were treated with L803mts (40 μM) or with CQ (30 μM) for 2 h. Cells were stained with LysoTracker Red and imaged by confocal microscopy. Representative images of 3 independent experiments are shown. NT, non-treated. C. MEF cells were treated with L803mts (40 μM) with or without vinblastine (20 mM) for 2 h. Cells were stained with LysoTracker Red and imaged by confocal microscopy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lysosomal acidification induced by GSK-3 inhibition [36,37]. We found that both the mTORC1/autophagy axis and endocytic trafficking are key players in GSK-3-regulated lysosomal acidification. In addition, we showed that functional TSC and Rab5 are required for the process of lysosomal acidification induced by GSK-3 inhibition.

We first showed that mTORC1 and autophagy jointly influence lysosomal acidification. We found that suppression of mTORC1 was not sufficient to promote lysosomal acidification in the absence of autophagy. Although autophagy is involved in lysosomal acidification, inhibition of GSK-3 resulted in acidification of lysosomes in the ATG5$^{-/-}$ cells. This unexpected result suggested the existence of an alternative autophagy-independent pathway that enables lysosomal acidification. It was possible that lysosomal biogenesis was induced by inhibition of mTORC1 and activation of TFEB, a master regulator of lysosomal biogenesis [86,87]; however, since treatment with cycloheximide did not prevent lysosomal acidification induced by L803mts, we concluded that lysosomal biogenesis was not the 'alternative' autophagy-independent pathway that induced lysosome acidification.

As lysosomes are located at the crossroads of autophagy and the endocytic pathways [11,34,52] and since acidification of late endosomes is independent of autophagosome fusion with lysosomes [88], we speculated that GSK-3 might control lysosomal acidification via the endocytic pathway. Indeed, our studies showed that GSK-3 inhibition accelerated early to late endosome conversion. We showed that expression of constitutively active Rab5, which impaired endosome trafficking, abolished the ability of GSK-3 inhibitors to induce lysosomal acidification. Furthermore, inhibition of GSK-3 increased the active pool of Rab5 as reflected by increased association with rabaptin-5 and the co-localization of these proteins at the endosome surface. The increased Rab5/Rab7 exchange was also manifested by recruitment of Rab7 to lysosomes in cells treated with GSK-3 inhibitor. The enhanced material internalization that was shown in cells treated with GSK-3 inhibitors was further evidence for activation of Rab5, which is required for endocytosis [63,67].

Our data implicates GSK-3 in internalization and down regulation of key signaling receptors. For example, GSK-3 activity attenuates receptors of the *N*-methyl-D-aspartate receptor family (NMDA/NMDAR) that play critical roles in synaptic activity and synaptic plasticity, a process that involves reduction in Rab5 activity [89,90]. Worth mentioning is the physical localization of GSK-3 with the late-endosomal compartments in response to Wnt-mediate signaling [91,92] that further demonstrates the tight relationship between GSK-3 and endosomal trafficking.

As a caveat, there is uncertainty in the field regarding the relationship between GSK-3 and TSC. Our results position GSK-3 as a negative regulator of TSC: Inhibition of mTORC1 in cells treated with the GSK-3 inhibitor required TSC, and, in addition, GSK-3 inhibition induced TSC translocation to the lysosome surface, a process previously shown to enable TSC inhibition of mTORC1 [85]. In this paradigm GSK-3 inhibition results in activation of TSC; this in turn inhibits mTORC1 to activate autophagy. Our results corroborate data that implicate GSK-3 as a positive regulator of mTORC1 presumably via inhibition of TSC [37,93–95]. Other studies, however, have shown the opposite [96–100]. Experimental conditions could explain these differences, particularly as the studies that suggest that GSK-3 is a negative regulator of TSC have examined the impact on TSC in the context of Wnt signaling [96]. Furthermore, an additional study showed that GSK-3 inhibits AMPK, again suggesting that GSK-3 positively regulates TSC by preventing its inhibition by AMPK [99].

In summary, the data shown here position GSK-3 as an important regulator of lysosomal acidification and provide further support for the use of selective GSK-3 inhibitors in treatment of pathological conditions

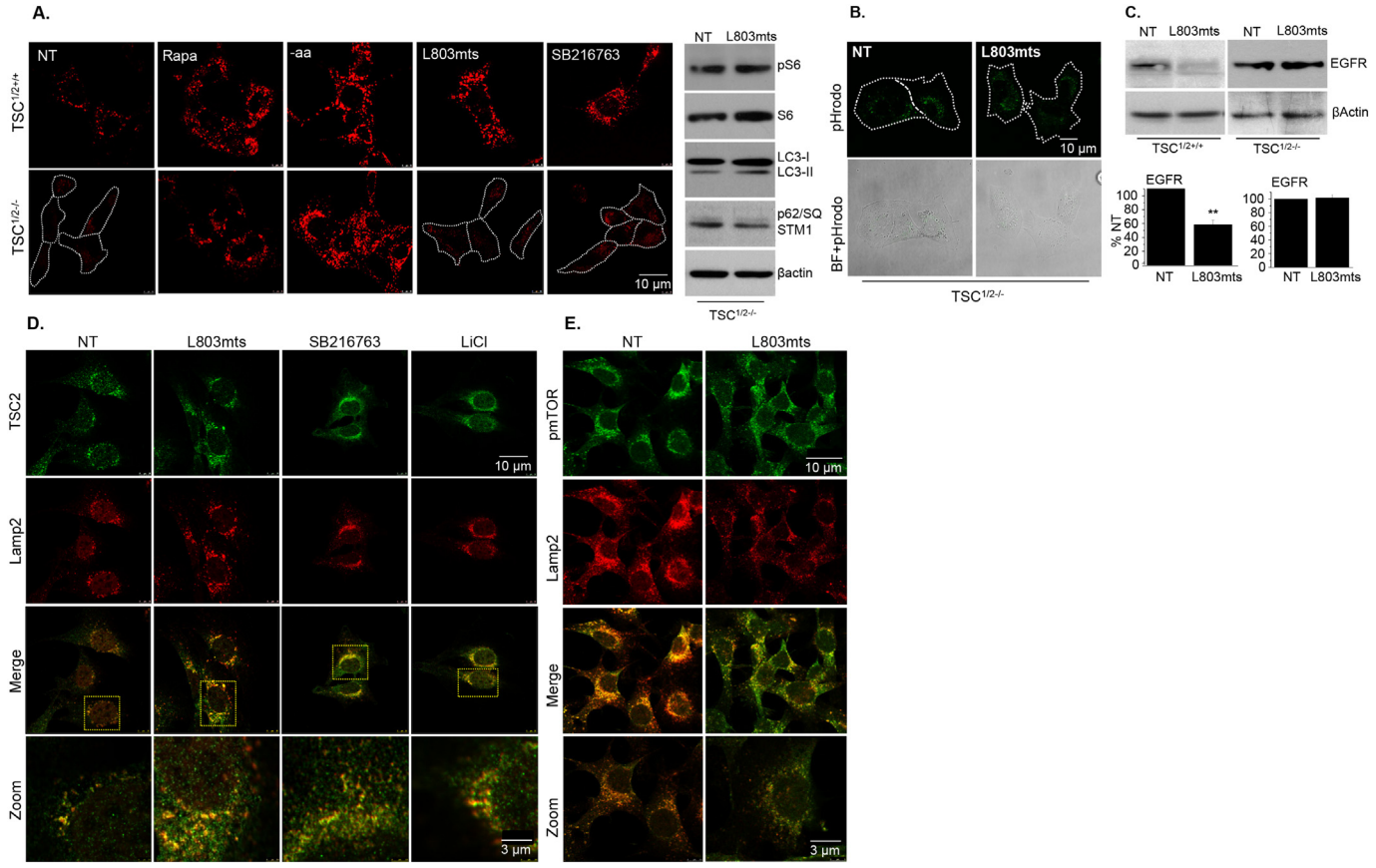

Fig. 7. Role of TSC in GSK-3-mediated lysosomal acidification. A. Left: MEF-TSC$^{+/+}$ or MEF-TSC$^{1/2-/-}$ cells were treated with rapamycin (100 nM), starved for amino acids (−aa), treated with L803mts (40 μM) or SB216763 (5 mM) for 2 h. Cells were stained with LysoTracker Red and imaged by confocal microscopy. Dashed lines indicate cells with low fluorescent signal Right: Phosphorylation of S6 and levels of LC3II, p62/SQSTM1 and b-actin were determined by immunoblot analysis in MEF-TSC$^{-/-}$ cells treated with L803mts as described. B. MEF cells were treated with L803mts (40 μM), SB216763 9 (5 μM), or LiCl (20 mM) for 2 h. Cells were fixed and co-immunostained with anti-TSC2 (green) and anti-Lamp2 (red) antibodies. Magnified regions (marked boxes) are shown in the lower panel. C. MEF cells were treated with L803mts as described. Cells were fixed and co-immunostained with anti-phospho mTOR(green) or anti-Lamp2 (red). Magnified images are shown at the lower panel. For all panels, representative images of 3 independent experiments are shown. NT, non-treated.

associated with impaired cellular homeostasis and cellular clearance.

4. Experimental procedures

4.1. Reagents

SB216763 and cycloheximide were from Sigma-Aldrich. Thapsigargin was from Enzo Life Sciences. Antibodies against phospho-S6K-1 (Thr^{389}), S6K-1, phospho-S6 (Ser$^{240/244}$), S6, LC3I/II, and phospho-TOR (Ser^{2448}) were from Cell Signaling Technologies. Antibodies against GSK-3α/β, β-actin, Lamp2, TSC2, Rab5, rabaptin-5, and EGFR were from Santa Cruz Biotechnology. Anti-p62/SQSTM1 was from Medical and Biological Laboratories, and anti-Rab7 was from Abcam.

4.2. Plasmids

The plasmids GFP-Rab7^{wt}, GFP-Rab7^{T22N}, GFP-Rab5^{WT}, GFP-Rab5^{Q79L} (referred to as GFP-Rab5^{CA}), and RFP-RILP were previously described [58,100]. GFP-EGFR plasmid was a kind gift from Dr. Yosef Yarden from the Weizmann institute of Science, Rehovot, Israel.

4.3. Cell culture, transfection, and treatments

Mouse embryonic fibroblasts (MEFs) deficient in *Presenilin* genes (MEF PS1/2$^{-/-}$; provided by Dr. Bart De Strooper, KU Leuven University, Belgium) and Chinese hamster ovary (CHO) *NPC1*-null cells (provided by Dr. Daniel Ory from Washington University, St. Louis, MO, USA) were grown in DMEM/F12 (1:1) supplemented with 10% FCS, 5 mM L-glutamine, and 1% penicillin-streptomycin. MEF cells deficient in *TSC1/2* (MEF TSC1/2$^{-/-}$) and matched MEF cells were generously provided by Dr. Kwiatkowski (Harvard Medical School, Boston, MA, USA). MEF cells deficient in *ATG5* (MEF ATG5$^{-/-}$) were provided by Dr. Zvulun Elazar (Weitzmann Institute, Rehovot, Israel). Both cell lines were grown in DMEM medium supplemented with 10% FCS, 5 mM L-glutamine, and 1% penicillin-streptomycin. For starvation we use Krebs-Ringer-HEPES buffer (0.4 mM KH_2PO_4, 0.1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 mM $NaHCO_3$, 3 mM HEPES, pH 7.4, 120 mM NaCl, 11 mM glucose) supplemented with serum as indicated. MEF cells were transfected with indicated constructs (2–3 μg) using the transfection reagent TransIT-X2 (Mirus Bio) according to the manufacturer's instructions. For silencing of *Rab7*, cells were transfected with 10 nM Rab7 siRNA or with a scrambled control siRNA (Dharmacon) using the transfection reagent Dharmafect (Dharmacon) according to the manufacturer's instructions.

4.4. Live cell imaging

To stain acidified lysosomes, cells grown on coverslips were incubated with 75 nM LysoTracker Red or 50 μM LysoSensor Green (both from Molecular Probes) for 15 min at 37 °C. The cells were washed with medium and were immediately imaged. For other experiments, the cells

were growth on coverslips and treated as indicated. Live cell images were taken using a 63.0 × 1.40 OIL UV objective lens on a laser scanning confocal microscope (Leica TCS-SP5 II) with spatial resolution of 50–70 nm. Images were generated using LAS-AF Lite software (Leica).

4.5. Immunofluorescent analyses

Cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature. After fixation, cells were permeabilized (0.2% Triton X-100), blocked with 3% bovine serum albumin, and incubated with indicated antibodies overnight at 4 °C. Cells were then washed three times in PBS and incubated with secondary Alexa488-conjugated or Alexa594-conjugated antibodies (Invitrogen-Molecular Probes) for 2 h at room temperature. CHO cells were incubated with 200 nM LysoTracker dye, fixed, and stained with Hoechst dye. Fixed cells were mounted (Fluoromount™ Aqueous Mounting Medium, Sigma Aldrich). Confocal images were acquired on the Leica TCS-SP5 II confocal microscope.

4.6. Endocytosis

Cells were transfected with GFP-EGFR plasmids. After 24 h, the cells were starved (0.1% FCS) and treated with L803mts for 2 h. EGF (50 ng/ml) was added. Aliquots of cells were PFA fixed at 5, 15, 30, and 60 min, and cells were imaged by confocal microscopy. For material internalization, cells treated or not treated with L803mts and were incubated with pHrodo Green dextran (200 μg/ml, Invitrogen-Molecular Probes) for 15 min. Live cells were imaged on the Leica TCS-SP5 II confocal microscope.

4.7. Gel electrophoresis and immunoblotting

Cells were collected and lysed in an ice-cold buffer G (20 mM Tris-HCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, 0.5 mM orthovanadate, 10 mM glycerol phosphate, 5 mM sodium pyrophosphate, 50 mM NaF, 1 mM benzaminidine, and protease inhibitors aprotenin, leupeptine, and pepstatin A). Cell extracts were centrifuged at 14,000 *g* for 20 min, and supernatants were collected. Equal amounts of protein (20–40 μg) were subjected to gel electrophoresis followed by western blot analysis using indicated antibodies. Analysis of β-actin levels demonstrated equal protein loading.

4.8. Statistical analysis

All experiments were repeated at least three times. The data are expressed as means ± standard deviations (SEM). Data were analyzed with Graph-pad Prism 8.1 software using the Student's *t*-test. Data were considered significant at $p < .05$.

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