TFEB phosphorylation on Serine 211 is induced by autophagy in human synovial fibroblasts and by p62/SQSTM1 overexpression in HEK293 cells

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Autophagy receptor p62/SQSTM1 signals a complex network that links autophagy-lysosomal system to proteasome. Phosphorylation of p62 on Serine 349 (P-Ser349 p62) is involved in a cell protective, antioxidant pathway. We have shown previously that P-Ser349 p62 occurs and is rapidly degraded during human synovial fibroblasts autophagy. In this work we observed that fingolimod (FTY720), used as a medication for multiple sclerosis, induced coordinated expression of p62, P-Ser349 p62 and inhibitory TFEB form, phosphorylated on Serine 211 (P-Ser211 TFEB), in human synovial fibroblasts. These effects were mimicked and potentiated by proteasome inhibitor MG132. In addition, FTY720 induced autophagic flux, LC3B-II up-regulation, Akt phosphorylation inhibition on Serine 473 but down-regulated TFEB, suggesting stalled autophagy. FTY720 decreased cytoplasmic fraction contained TFEB but induced TFEB in nuclear fraction. FTY720-induced P-Ser211 TFEB was mainly found in membrane fraction. Autophagy and VPS34 kinase inhibitor, autophinib, further increased FTY720-induced P-Ser349 p62 but inhibited concomitant expression of P-Ser211 TFEB. These results suggested that P-Ser211 TFEB expression depends on autophagy. Overexpression of GFP tagged TFEB in HEK293 cells showed concomitant expression of its phosphorylated form on Serine 211, that was down-regulated by autophinib. These results suggested that autophagy might be autoregulated through P-Ser211 TFEB as a negative feedback loop. Of interest, overexpression of p62, p62 phosphorylation mimetic (S349E) mutant and phosphorylation deficient mutant (S349A) in HEK293 cells markedly induced P-Ser211 TFEB. These results showed that p62 is involved in regulation of TFEB phosphorylation on Serine 211 but that this involvement does not depend on p62 phosphorylation on Serine 349.

Introduction

Two proteolytic systems, autophagy and ubiquitin proteasome system, have been reported to be interactive due to p62/SQSTM1 that participates in both processes [1]. Complexity of this interaction was further documented by the discovery that proteasome itself is degraded by autophagy through the process of proteaphagy [2,3]. It has been suggested that p62 domain that binds to proteasome have a dual role: to target substrates to the proteasome or/and to target proteasome to autophagy [2]. TFEB is a master inducer of lysosome expression and regulation, as well as of lysosome-associated processes like autophagy, and p62 is one of the TFEB induced target genes [4]. Park et al. [5] showed recently that TFEB also induces p62 phosphorylation on Serine 349 (P-Ser349 p62). Inhibitory phosphorylation of TFEB is mediated by mTORC1 on Serine 211 (P-Ser211 TFEB) [6–8] and by Akt, on Serine 467 [9]. These phosphorylations prevent TFEB nuclear localization and activation of CLEAR...
Experiments were repeated at least three times with cells from at least three different patients. Plates were frozen at 3 h. At the end of experiments culture medium was removed, plates cooled on ice and cold lysis buffer [27] 88899-55-2, Cat BML-CM110, Enzo Life Sciences) for 30 min and then stimulated with FTY720 for additional.

Material and methods
Cell isolation and culturing
Human synovial fibroblasts were isolated and cultured as explained previously [25]. Briefly, cells (1 × 50^3) were seeded into 24 well plates containing 0.5 ml of DMEM (Cambrex Bio Science), supplemented with 10% FCS (Biowest), 1-glutamine (2 mM), streptomycin (100 mg/ml) and penicillin (100 U/ml) (BioWhittaker). After 24 h cells were treated with FTY720 (CAS 1623559-56-0, Cat SML0700, Sigma – Aldrich) and/or MG132 (CAS 133407-82-6, Cat 474790, Sigma – Aldrich) and autophinib (CAS 164443-47-9, Cat 6324, Tocris Bioscience) for additional 3–24 h. To determine autophagic flux [26] cells were pretreated with Bafilomycin A1 (CAS 88899-55-2, Cat BML-CM110, Enzo Life Sciences) for 30 min and then stimulated with FTY720 for additional 3 h. At the end of experiments culture medium was removed, plates cooled on ice and cold lysis buffer [27] added. Plates were frozen at −80°C or −20°C and lysed cells scraped on ice. Cell extracts were stored at −20°C. Experiments were repeated at least three times with cells from at least three different patients.

DNA transfection
HEK293 cells (50 000 cells/well) were seeded in 24 well plates in 0.5 ml of DMEM supplemented with 10% FCS, 1-glutamine, streptomycin, and penicillin, and cultured for 24 h. Then, transfection was done with 0.5–2 μg/ml of pEGFP-N1-TFEB [7], 2 μg/ml of p62 [21], p62-HA, p62-HA phosphorylation mimetic mutant S349E, p62-HA phosphorylation defective mutant S349A [19,21], pEGFP-C1 (Clontech) and 1 μg/ml pLC3B-GFP [28], by the use of linear polyethylenimine (PEI)(MW 25,000) (Polysciences, Inc., Warrington, PA), for 3 h. Then, culture medium was changed and cells were treated or not with autophinib for additional 24 h. pEGFP-N1-TFEB was a gift from Dr Shawn Ferguson (Addgene plasmid # 38119; http://n2t.net/ addgene:38119; RRID:Addgene_38119) [7]. EGFP-LC3 was a gift from Karla Kirkegaard (Addgene plasmid # 11546; http://n2t.net/addgene:11546; RRID:Addgene_11546) [28]. Stock solution of PEI was made in demineralized water (1 mg/ml), filter sterilized and stored at 4°C or −20°C. For one transfection 1 μg of DNA was dissolved in 50 μl of DMEM and 15 μl of PEI stock solution was added. Following vigorous vortexing, solution was left for 30 min at room temperature before gently addition to the cells.

Western blotting
Protein expression in total cell extracts were determined by Western blotting as explained previously [25], by the use of following primary antibodies: p62/SQSTM1 (P0067), Anti-LC3B (L7543), GAPDH (G9545), α-Tubulin (T6074), Sigma–Aldrich, Anti-Phospho-p62 (SQSTM1) (Ser351) (M217-3), MBL, Phospho-Akt (coordinated lysosomal expression and regulation) bearing genes [7]. P-Ser211 TFEB has cytoplasmic localization, and interacts with 14-3-3 that masks its nuclear localization signal [7]. TFEB is activated through dephosphorylation of Serine 211 by calcineurin [10] and PP2A [11].

Complex signaling of p62 is involved in several diseases, including Paget disease of bone [12,13]. Autophagy has protective effect on chondrocytes [14] and TFEB is proposed as therapeutic target for osteoarthritis [15]. TFEB is down-regulated in osteoarthritis and its re-activation induces autophagy and protect chondrocytes from apoptosis [15]. Of interest, P-Ser349 p62 was recognized as protective in collagen-induced arthritis [16]. However, in rheumatoid arthritis patients, a pathogenic subset of CD4+T cells, with increased level of autophagy was found, leading to T-cell hyperactivation [17,18]. In Alzheimer’s disease (AD), P-Ser349 p62 is detected in brain [19]. However, overexpression of p62 stimulates autophagy and plays protective role in some neurodegenerative diseases [20]. Therefore, further knowledge on p62 and P-Ser349 p62 tuning may help combating disease in which p62 is involved.

We have shown previously that P-Ser349 p62 appears and is quickly degraded during human synovial fibroblast autophagy [21]. In that work we showed that proteasome inhibition markedly stabilizes P-Ser349 p62 and that concomitant inhibition of autophagy increases this effect. Previously, Roczniak-Ferguson et al. [7] showed that inhibition of autophagy can cause TFEB nuclear localization. These results suggested the existence of a negative feedback loop(s) that regulate autophagy. Recently, the involvement of p62 in the existence of both positive and negative feedback in autophagy was shown [22].

In the work presented here we studied the effect of fingolimod (FTY720), a medication used for multiple sclerosis treatment [23] that suppresses murine experimental arthritis [24], on human synovial fibroblast autophagy.
Microscopy
FTY720-induced vacuoles in alive, 24 h treated human synovial fibroblasts were observed and photos captured by phase contrast enhanced light microscopy using a digital sight camera (2MV) under the inverted microscope Eclipse TS100 (Nikon Instruments Inc., Melvile, NY, U.S.A.), phase contrast objectives and NIS-Elements Basic Research software (Nikon Instruments Inc.). Fluorescence microscopy was done on alive HEK293 cell, 24 h after transfection, by Leica DM IL (Leica) and LAS software, version 4.11 (Leica).

Acridine orange staining
Human synovial fibroblasts were cultured in the absence or presence of FTY720 (10 µM) for 24 h. Acridine orange was then added to the cells at concentration 2 µg/ml, for additional 3 h and phase contrast improved light microscopy on alive cells performed. Stock solution of acridine orange was made in ethanol and stored at −20°C.

Subcellular fractionation
Subcellular fractionation of 1.5 × 10⁶ human synovial fibroblasts was done as explained earlier [27,29]. To obtain membrane fraction insoluble pellet was boiled in 10% glycerol, 2% SDS, and 50 mM Tris–HCl (pH 7.5) for 10 min [30].

Statistical analysis
Mann–Whitney test and Student’s t-test were used to obtain p values. A value of P < 0.05 was considered as statistically significant.

Results
FTY720 induces cytoplasmic vacuoles, LC3B-II up-regulation and autophagic flux in human synovial fibroblasts
Human synovial fibroblasts were cultured in the presence or absence of FTY720 (Figure 1A,B). FTY720-treated human synovial fibroblasts showed marked cytoplasmic vacuolization, observed by phase contrast improved light microscopy after 24 h (Figure 1A). Appearance of these vacuoles was visible after 2–3 h of treatment already (results not shown). Addition of acridine orange in culture medium of 24 h FTY720-treated cells, for additional 3 h, showed orange staining accumulation in the subset of these vacuoles (Figure 1B). These results showed that a subset of FTY720-induced vacuoles is acidic and suggested that FTY720 may induce synovial fibroblasts autophagy. To determined effects of FTY720 on autophagic flux, synovial fibroblasts were pretreated with different concentrations of Bafilomycin A1, then stimulated with different concentrations of FTY720 and effects on LC3B-II expression was monitored by Western blot (Figure 1C). Results obtained with synovial fibroblasts from three different patients showed that, after 3 h of treatment, FTY720 significantly increased autophagic flux when cells were pretreated with 20 nM Bafilomycin A1 and stimulated with 5 µM FTY720 (Figure 1C, line 7) but not when cells were pretreated with 40 nM Bafilomycin A1 (Figure 1C, line 10). These results showed that FTY720 can increase autophagic flux in synovial fibroblasts. However, these results also revealed that FTY720 effect is rapidly saturated.

FTY720 down-regulates Akt phosphorylation on Serine 473
We have tested Akt phosphorylation on Serine 473 in human synovial fibroblasts treated with different concentrations of FTY720 (2.5–10 µM) for 3 h (Figure 2). Serum deprivation was used as a negative control. Results showed that FTY720 significantly down-regulated Akt phosphorylation in a concentration dependent manner, in the presence of serum (Figure 2, lines 3, 5 and 7) and showed statistically significant additive effect to serum deprivation induced Akt dephosphorylation (Figure 2, line 6). However, during serum deprivation higher concentration of FTY720 (10 µM) induced cell death (results not shown). In parallel with inhibition of Akt
Figure 1. FTY720 induces cytoplasmic vacuoles and autophagic flux in human synovial fibroblasts.
(A) Human synovial fibroblasts were treated or not with FTY720 (10 μM) for 24 h. (B) Cells were treated or not with FTY720 (10 μM) for 24 h and then acridine orange (2 μg/ml) was added for additional 3 h. Photos were taken by phase contrast improved light microscopy. (C) For autophagic flux analyses cells were preincubated with Bafilomycin A1 for 30 min and then treated with FTY720 for additional 3 h. Western blots show LC3B-I, LC3B-II, p62 and GAPDH expressions in total cell extracts. Graphs show average of LC3B-II expression (%) calculated from three experiments done with synovial fibroblasts from three different OA patients. a*, b* and c* are significantly higher than control. b* is significantly higher than a* and c*. d* is significantly higher than a*.

Figure 2. FTY720 induces Akt1 de-phosphorylation on Serine 473.
Human synovial fibroblasts were treated with different concentration of FTY720 (2.5–10 μM), in the presence or absence of serum. Western blots show Akt phosphorylated on Serine 473, LC3B-I, LC3B-II p62 and GAPDH expressions in total cell extracts. Graphs show average of p-Akt (473) and LC3B-II expression (%) calculated from three experiments done with synovial fibroblasts from three different OA patients. a*–f* are significantly lower than control; e* is significantly lower than a* and d*. g*–j* are significantly higher than control; i* is significantly higher than g* and h*; j* is significantly higher than h*.

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phosphorylation on Serine 473, FTY720-induced accumulation of LC3B-II (Figure 2, lines 5 and 7). FTY720 (5 μM) and serum deprivation had statistically significant additive effect on LC3B-II up-regulation (Figure 2, line 6). These results showed that FTY720 down-regulated Akt phosphorylation on Serine 473.

Coordinated expression of p62 and P-S349 p62 with P-S211 TFEB in human synovial fibroblasts upon FTY720 treatment

Since FTY720 induced autophagic flux, LC3B-II and inhibition of Akt phosphorylation on Serine 473, all characteristics of autophagy, we have tested FTY720 effects on TFEB, a master protein that coordinate expression of autophagy and lysosomal genes, as well as on P-S349 p62 for which we have shown to be involved in human synovial autophagy [21]. Results showed that FTY720 increased both p62 and P-S349 p62 but down-regulated TFEB (Figure 3A, line 2). Furthermore, FTY720 induced TFEB inhibitory form P-Ser211 TFEB (Figure 3A, line 2). Smear like TFEB band detected in control cells suggested protein posttranslational modification (Figure 3A, line 1). Coordinated expression of p62, P-Ser349 p62 and P-Ser211 TFEB was both potentiated (Figure 3A, lines 3–5) and mimicked (Figure 3A, lines 7–8) with proteasome inhibitor MG132. Autophinib, an autophagy and VPS34 (vacuolar protein sorting 34) kinase inhibitor, increased FTY720-induced P-Ser349 p62 (Figure 3A, lines 9–10) but, in contrast with MG132, inhibited FTY720-induced P-Ser211 TFEB (Figure 3A, line 10). These results suggested that P-Ser211 TFEB expression depends on autophagy activity.

FTY720 changes TFEB subcellular localization in human synovial fibroblasts

To follow the fate of TFEB during FTY720 treatment, we have isolated proteins by subcellular fractionation and performed Western blots using antibodies for TFEB or P-Ser211 TFEB (Figure 3B). Results showed that TFEB is localized in the cytoplasm of control cells (Figure 3B, line 1). In FTY720-treated cells less TFEB was found.
in the cytoplasmic fraction (Figure 3B, line 2) while TFEB appeared in the nuclear fraction (Figure 3B, line 6). No P-Ser211 TFEB was found in control cells (Figure 3B, lines 1, 5 and 9) while in FTY720-treated cells P-Ser211 TFEB appeared in cytoplasmic (Figure 3B, line 2) and mostly in membrane fraction (Figure 3B, line 10). Proteasome inhibitor MG132 had both similar (Figure 3B, lines 4, 8 and 12) and additive (Figure 3B, lines 3, 7 and 11) effect with FTY720 on TFEB subcellular localization.

**Concomitant expression of TFEB-GFP phosphorylated on Serine 211 (P-Ser211 TFEB-GFP) in HEK293 cells overexpressing TFEB-GFP is autophosphorylation sensitive**

Because proteasome inhibition increased, but autophagy inhibition decreases FTY720-induced P-Ser211 TFEB (Figure 3), we have tested hypothesis that P-Ser211 TFEB expression depends on autophagy. Different amount of EGFP tagged TFEB (pEGFP-N1-TFEB) [7] were transfected to HEK293 cells (Figure 4). After 3 h of transfection, cells were washed and treated or not with autophinib for additional 24 h. TFEB and P-Ser211 TFEB were determined in total cell extracts by western blotting (Figure 4). Results showed that pEGFP-N1-TFEB-transfected cells expressed TFEB-GFP and, in parallel, P-Ser211 TFEB-GFP (Figure 4, lines 2–4) and mostly in membrane fraction (Figure 3B, line 10). Proteasome inhibitor MG132 had both similar (Figure 3B, lines 4, 8 and 12) and additive (Figure 3B, lines 3, 7 and 11) effect with FTY720 on TFEB subcellular localization.

**Overexpression of p62 induces P-Ser211 TFEB**

Concomitant expression of p62 and P-Ser211 TFEB in FTY720- and MG132-treated human synovial fibroblasts suggested that p62 may be involved in P-Ser211 TFEB expression. To test p62 effect on P-Ser211 TFEB we have transfected p62 [31] into HEK293 cells and determined P-Ser211 TFEB in total cell extracts by Western blot (Figure 5A). Results showed that p62 transfected cells (Figure 5A, line 2) but not GFP transfected cells (Figure 5A, line 3) and non-transfected cells neither (Figure 4A, line 1) markedly expressed P-Ser211 TFEB.
These results showed that p62 is involved in TFEB phosphorylation on Serine 211. To test if p62 Serine349 phosphorylation is involved in this phenomena, we have compared phosphorylation mimetic p62 mutant (S349E) and phosphorylation deficient p62 mutant (S349A) with wild type, HA-tagged p62 (p62-HA) [19].

Figure 4. Serine 211 phosphorylation of overexpressed TFEB is autophinib sensitive.
HEK293 cells were transfected with increasing concentration of pEGFP-N1-TFEB (0.5–2 mg/ml). After 3 h post-transfection medium was changed and cells cultured in the presence or absence of autophinib for additional 24 h. Western blots show TFEB-GFP, P-Ser211 TFEB-GFP, p62, LC3B and GAPDH expressions in total cell extracts.

Figure 5. Overexpression of p62 induces P-Ser211 TFEB.
(A) HEK293 cells were transfected with 2 μg/ml of p62 or pEGFP-C1. (B) HEK293 cells were transfected with 2 μg/ml of p62-HA, p62 phosphorylation mimetic mutant S349E, p62 phosphorylation deficient mutant S349A or pEGFP-C1. Western blots show P-Ser211 TFEB, p62, P-Ser349 p62, GFP and GAPDH expressions in total cell extracts. (C) HEK293 cells were transfected with EGFP tagged LC3 (EGFP-LC3) or co-transfected with p62-HA, p62 phosphorylation mimetic mutant S349E and p62 phosphorylation deficient mutant S349A. Photos show EGFP-LC3 sparkles taken by fluorescence microscope. Graf represent average number of sparkles counted in 4 × 20 transfected cells for each treatment. a* is significantly higher than EGFP-LC3 transfected cells.
Results showed that, as p62-HA, both mutants were also able to markedly induce P-Ser211 TFEB (Figure 5B, lines 3–5) as well as to form sparkles when co-transfected with pLC3B-GFP (Figure 5C). These results showed that the involvement of p62 in TFEB phosphorylation on Serine 211 does not depend on p62 phosphorylation on Serine 349.

**Discussion**

Fingolimod (FTY720) is an immunomodulatory drug used against multiple sclerosis, it favors remyelination [32] and also ameliorates brain injury [33]. FTY720 has agonistic and transient anti-agonistic sphingolipid-like properties. Sphingolipids down-regulate cell surface transporters for amino acids and glucose, thus acting as starvation mimetics [34] and FTY720 was shown to induce cancer cell starvation, cell death and homeostatic autophagy [35]. In the presented work we showed that FTY720 induced stalled-like autophagy in human synovial fibroblast. FTY720 induced autophagic flux, LC3B-II up-regulation, Akt1 dephosphorylation on Serine 473 and P-Ser349 p62, all suggesting autophagy [21,36]. Akt phosphorylates TFEB on Serine 467 and prevent its nuclear localization, while Akt inhibition induces TFEB activation [9]. However, FTY720 also induced marked up-regulation of p62 and TFEB down-regulation suggested stalled autophagy. Stabilization of p62, despite the LC3B-II up-regulation has been shown previously and suggested as stalled autophagic flux [37]. However, overexpression of p62 was also shown to stimulate autophagy and play protective role in some neurodegenerative diseases [20]. Increased p62 expression and blocked autophagy were shown previously to be induced by FTY720 in mantle cell lymphoma cell lines [38]. In these cells FTY720 induced lysosomal enlargement, lysosomal membrane permeabilization and translocation of lysosomal hydrolases in the cytosol, resulting in cell death [38]. FTY720-induced autophagy inhibition and p62 accumulation, followed by cell death, were also detected in rat pancreatic stellate cells [39]. However, FTY720 was also shown to protect from ischemic brain damage in mice, through neuronal autophagy inhibition [40]. In our work, FTY720 did not induce cell death in human synovial fibroblasts cultured in standard serum containing medium and even protected cells from apoptotic agent BAY11-7085 (results not shown). This effect may involve FTY720 ability to induce P-Ser349 p62. Others and we have shown previously that both mouse p62 phosphorylated on Serine 351 [41] and human P-Ser349 p62 have protective role against cell death [21].

Our results showed that FTY720 markedly induced LC3B-II. LC3B-II participates in the formation of autophagosome [42] and it is the most accepted marker of autophagy process [43]. However, although we showed here that FTY720 is able to induce autophagic flux in human synovial fibroblasts in early hours of treatment, this effect is rapidly saturated, further suggesting that FTY720-induced autophagy is stalled. Furthermore, we showed here that FTY720 induced cytoplasmic vacuolization in human synovial fibroblasts. The number of vacuoles increased with autophinib (inhibitor of both Vps34 phosphatidylinositol 3-kinase and autophagy) treatment (results not shown). Of interest, down-regulation of Vps34 induces similar vacuolization [44]. Endogenous TFEB has been difficult to analyze because of low level of TFEB protein in many cell types [45]. In this work TFEB was detected by western blot in human synovial fibroblast extracts as a smear like band suggesting post translational modification, such as glycosylation, that will be further tested. We showed that FTY720 induced P-Ser349 p62, involved in autophagy, but down-regulated total TFEB. However, by subcellular fractionation, we found TFEB, apart from cytoplasmic fraction, in the nuclear fraction of FTY720-treated synovial fibroblasts, suggesting post-autophagic effect. In addition, FTY720 induced TFEB inhibitory form (P-Ser211 TFEB) [7] that coincidence with p62 and P-S349 p62 expressions. These results suggested a negative feedback in which autophagy is autotuned through P-Ser211 TFEB. To test this hypothesis, we have performed experiments with autophagy inhibitor and showed that lack of autophagy had inhibitory effect on FTY720-induced P-Ser211 TFEB expression. Furthermore, expression of TFEB-GFP in HEK293 cells was followed by autophinib sensitive expression of P-Ser211 TFEB-GFP. These results showed that autophagy is involved in P-S211 TFEB expression and suggest existence of a negative feedback through which autophagy can be tuned.

Discovery of TFEB [46] and its functions [47] lightened our understanding of autophagy process [6–8,47]. Expression of TFEB-GFP in HeLa-M cells resulted in dominant cytoplasmic localization of TFEB [7]. Intrigued by the fact that in lysosomal storage disorders TFEB accumulates in the nucleus [48], authors inhibited lysosomal function with chloroquine and bafilomycin A and showed that in these conditions TFEB is gaining nuclear localization, followed by TFEB down-regulation [7]. These results support existence of negative feedback in which autophagy is necessary for TFEB phosphorylation.
Very recently, the feedforward loop between p62 and TFEB was observed during cardiac macroautophagy induced by proteasome inhibition [49]. p62 — TFEB relationship has been observed in human epidermal keratinocytes [50]. In that work was shown that ultraviolet (UV) radiation causes TFEB dephosphorylation, TFEB nuclear localization as well as p62 transcription and accumulation [50]. Existence of positive and negative feedback in autophagy was shown previously for p62 [22]. By binding to Keap1, P-S349 p62 activates Nrf2 that in return increases expression of both full length p62 and its splice variant that has no Keap1 interacting site [22]. As a result, the splice variant is making a negative feedback on Nrf2 expression [12,22]. In this work we showed that expression of P-Ser211 TFEB, an inhibitory form of TFEB, depended on autophagy. Furthermore, we showed here that overexpression of both p62 and P-Ser349 p62 induced P-Ser211 TFEB. This effect, however, was also observed with p62 phosphorylation defective mutant (S349A), showing that p62 involvement in TFEB phosphorylation on S211 does not depended on p62 phosphorylation on Serine 349. Of interest, p62 has been found to be associated with kinase activity and it has been proposed that p62 itself might be a Ser/Thr protein kinase or that p62 is associated to other Ser/Thr protein kinase [51,52]. We showed here that TFEB phosphorylation on S211 was down-regulated with autophinib, a VPS34 (class III PI3K) kinase inhibitor. Inhibition of VPS34 kinase, belonging to class III PI3K, showed that this kinase is involved in the activity of Ser/Thr kinase SGK3 [53,54]. Recently, it was shown that p62 induces ULK1 phosphorylation through increased AMPK–ULK1 interaction [55]. Further work is necessary to reveal if p62 is directly or indirectly involved in TFEB phosphorylation on Serine 211.

**Data Availability**

All supporting data are included within the main article.

**Competing Interests**

The authors declare that there are no competing interests associated with the manuscript.

**Funding**

This work was supported by funding from Fonds d’Investissement pour la Recherche Scientifique (FIRS), CHU, Liège, Belgium.

**CRediT Author Contribution**

Biserka Relic: Conceptualization, Formal analysis, Investigation, Methodology, Writing — review and editing. Celine Deroyer: Investigation, Methodology, Writing — review and editing. Olivier Malaise: Investigation, Methodology. Zelda Plener: Formal analysis, Methodology. Philippe Gillet: Formal analysis, Methodology. Dominique de Seny: Funding acquisition, Investigation, Methodology, Writing — review and editing. Michel G. Malaise: Conceptualization, Funding acquisition, Investigation, Writing — review and editing.

**Acknowledgements**

We would like to thank to Dr Claire Josse for useful discussions and to Audrey Hoffmann and Lisa Beaudry for some aspects of the technical work.

**Abbreviations**

GFP, green fluorescent protein; mTORC1, mammalian target of rapamycin complex 1; PP2A, protein phosphatase 2A; P-Ser211 TFEB, TFEB phosphorylated on Serine 211; P-Ser349 p62, Phosphorylation of p62 on Serine 349; SGK3, serum- and glucocorticoid-inducible kinase 3; TFEB, transcription factor EB; VPS34, vacuolar protein sorting 34.

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