Resveratrol promotes lysosomal function via ER calcium-dependent TFEB activation to ameliorate lipid accumulation

Rong Shao\textsuperscript{1}, Jiahui Shi\textsuperscript{1}, Kaili Du\textsuperscript{1,2}, Na Wang\textsuperscript{1,2}, Weijie Cai\textsuperscript{1}, Siyu Liu\textsuperscript{1}, Zongxian Ding\textsuperscript{1}, Yihan Wang\textsuperscript{1}, Dan Li\textsuperscript{1,2} \#

\textsuperscript{1} Collaborative Innovation Center of Yangtze River Delta Region Green Pharmaceuticals, College of Pharmaceutical Sciences, Zhejiang University of Technology, Hangzhou 310014, China

\textsuperscript{2} Department of Molecular, Cellular, and Developmental Biology, University of Michigan, 3089 Natural Science Building (Kraus), 830 North University Avenue, Ann Arbor, MI 48109, USA

Running Title: Resveratrol activates PP2A and TFEB-dependent lysosomal function to ameliorate lipid accumulation

Key words: Resveratrol, Lysosome, TFEB, PP2A

\# Correspondence should be addressed to:

D. L. (Email: lidan@zjut.edu.cn)
Abstract:
Abnormal lipid accumulation is associated to the development of metabolic diseases such as hepatic steatosis and lipid storage diseases. Pharmacological agents that can attenuate lipid accumulation therefore have therapeutic potentials for these diseases. Resveratrol (RSV), a natural active substance found in fruits and nuts, has been reported to effectively reduce the intracellular lipid accumulation, but the underlying mechanisms of RSV remain elusive. Here, we show that RSV triggers an endoplasmic reticulum (ER)-Ca²⁺ signaling that activates transcriptional factor EB (TFEB), a master transcriptional regulator of autophagic and lysosomal biogenesis. Moreover, RSV activates protein phosphatase 2A (PP2A), which binds and dephosphorylates TFEB, promoting its nuclear translocation and the expression of TFEB target genes required for autophagosome and lysosomal biogenesis. Notably, genetic inhibition of TFEB significantly ameliorates RSV-mediated lipid clearance. Taken together, these data link RSV-induced ER calcium signaling, PP2A and TFEB activation to promote autophagy and lysosomal function, by which RSV may trigger a cellular self-defense mechanism that effectively mitigate lipid accumulation commonly associated with many metabolic diseases.

Abbreviation:
ANOVA, analyses of variance; AMPK, adenosine 5’-monophosphate (AMP)-activated protein kinase; BAF-A1, bafilomycin A1; CLEAR, coordinated lysosomal expression and regulation; DCFH-DA, 2ʹ,7ʹ-dichlorofluorescin diacetate; ER, endoplasmic reticulum; GPN, glycyl-L-phenylalanine 2-napthylamide; KO, knockout; LAMP1, lysosomal associated membrane protein 1; LSD, lysosomal storage diseases; NAC, N-acetylcysteine; NRF2, nuclear factor -E2-related factor 2; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OA, okadaic acid; PPP2/PP2A, protein phosphatase 2A; Q-PCR, quantitative real time PCR; ROS, reactive oxygen species; RSV, resveratrol; SA, stearic acid; SQSTM1/p62, sequestosome 1; TFEB, transcription factor EB; TG, thapsigargin; mTOR, mammalian target of rapamycin kinase; WT, wild type.
Introduction

Macro-autophagy is a cellular process involving lysosomal degradation of cytosolic components through formation of a double membrane structure (autophagosome) and its fusion to lysosome (autophagolysosome) [1]. Amino acids and other small molecules produced by lysosome degradation can be reused or generate energy. One of the main functions of autophagy is to maintain cell survival when cells are threatened by stressful death, and plays an important role in maintaining the metabolic homeostasis of cells [2]. Impairment of autophagy-lysosome pathway is associated with metabolic disorders and aging. In metabolic syndromes such as obesity and fatty liver disease [3, 4], excessive nutrients challenge the adaptive response capacity of degradative autophagy-lysosome machinery. Undigested macromolecules (ie. lipids, proteins) and impaired organelle turnover compromise metabolic activity, provoking intracellular stresses, and exacerbate collateral defects in insulin action or other metabolic pathologies. Therefore, pharmacological interventions that enhance autophagic and lysosomal function are emerging as a promising strategy to ameliorate metabolic symptoms and promote longevity.

The transcriptional factor EB (TFEB) is a major regulator of autophagic and lysosomal biogenesis [5, 6]. TFEB belongs to MITF/TFE family of basic helix-loop-helix/leucine zipper transcriptional factors and binds to the “coordinated lysosomal expression and regulation (CLEAR)” motifs located in the promoter region of a variety of autophagic and lysosomal genes [6, 7]. TFEB activity is regulated by phosphorylation [8-10], which keeps TFEB inactive in the cytoplasm; in contrast, dephosphorylated TFEB travels to the nucleus to activate transcriptional target genes [11]. Several pathways reportedly control TFEB activity under different conditions. Under starvation, the phosphorylation of TFEB is suppressed by mammalian target of rapamycin kinase (mTOR) inhibition [12]. Under oxidative stress, TFEB is activated via a lysosomal Ca\(^{2+}\)-dependent mechanism independent of mTOR [13]. Notably, overexpression of TFEB homolog HLH-30 in Caenorhabditis elegans can increase lifespan through possible induction of autophagy [14]. Hence, TFEB agonists are potential therapeutic intervention for diseases in which dysfunctional autophagy and lysosome has been implicated.
Natural compounds in food are known to be good potential drug targets, but the underlying mechanisms are not clear [15]. In the present study, we demonstrate that resveratrol (RSV) (Fig. 1a), an active natural substance from fruits and peanuts, induces a Ca\(^{2+}\) signaling from endoplasmic reticulum (ER) and promotes autophagic and lysosomal function through protein phosphatase 2A (PP2A) – mediated TFEB activation. Moreover, genetic inactivation of TFEB impairs RSV function of ameliorating lipid accumulation, suggesting that TFEB is required in RSV- mediated lipid clearance.

**Results**

**Ca\(^{2+}\) is required for RSV-induced TFEB nuclear translocation**

First, we investigated the effect of RSV on TFEB activation in HeLa cells stably expressing GFP-TFEB[16]. At the concentration of 300 µM, but not at a lower concentration (100 µM), RSV treatment for 4 h induced a five-fold increase of TFEB translocation from the cytosol to the nucleus (Fig. 1b, c). Consistently, RSV (300 µM, 4 h) induced endogenous TFEB nuclear translocation in HeLa wild-type (WT) cells (Fig. 1d, e).

We next analyzed the mRNA expression of TFEB target genes, including a selected list of genes involved in autophagy and lysosome using quantitative real time PCR (Q-PCR). RSV is a known antioxidant and transcriptional factor - nuclear factor -E2-related factor 2 (NRF2) inducer [17]. Hence, the transcriptional expression levels of *HO-1* and *NQO1* gene (NRF2 downstream genes) were used as a positive control. Upon RSV treatment (300 µM, 12 h), the expression levels of lysosomal genes (*CTSD, NEU1 and LAMP1*) and autophagic genes (*WIP1*) were significantly increased in HeLa cells (Fig. 1f). Collectively, in agreement with recent studies [18], RSV induces TFEB nuclear translocation and the expression of TFEB target genes.

As intracellular Ca\(^{2+}\) reportedly involves in TFEB activation [11, 13], we then assessed the dependence of Ca\(^{2+}\) in TFEB activation by RSV using specific Ca\(^{2+}\) chelators. Remarkably, pretreatment with BAPTA-AM (10 µM, 1 h), a commonly used Ca\(^{2+}\) chelator, significantly blocked RSV (300 µM, 4 h)-induced TFEB nuclear translocation in GFP-TFEB
(Fig. 2a, b) and WT HeLa cells (Fig. 2c, d), suggesting that Ca\(^{2+}\) is required in RSV-induced TFEB activation.

RSV also reportedly induces low level of ROS [19], which involves in many functions of RSV [19]. To evaluate the effect of ROS on RSV-mediated TFEB activation, we exposed HeLa GFP-TFEB cells to N-acetylcysteine (NAC), a commonly used antioxidant. Following coapplication of NAC (5 mM) for 4 h, TFEB nuclear translocation induced by RSV was not affected (Fig. 2e, f), demonstrating that RSV-mediated TFEB activation is ROS-independent.

**ER calcium store contributes to TFEB activation by RSV**

Our data indicate that RSV-induced TFEB nuclear translocation requires cytosolic calcium. As known that lysosomes, mitochondria and ER are the major compartmentalized Ca\(^{2+}\) stores in cells [20], the mechanistic links between chemically induced cytosolic Ca\(^{2+}\) and TFEB activation lead us to consider the discrete Ca\(^{2+}\) sources as a potential specificity determinant of RSV-induced TFEB activation. We next investigated the determinant Ca\(^{2+}\) sources in RSV-induced TFEB activation. In HeLa GFP-TFEB cells, RSV-induced TFEB nuclear translocation was blocked by pretreatment with thapsigargin (TG) (300 nM, 30 min), a specific inhibitor of ER Ca\(^{2+}\) ATPase SERCA pump that is commonly used to deplete ER Ca\(^{2+}\) stores [21] (Fig. 3a, b). In contrast, RSV-induced TFEB nuclear translocation was not affected by 30 min pretreatment of glycyl-L-phenylalanine 2-napthylamide (GPN) (200 µM), a lysosome-disrupting agent to deplete lysosome-specific Ca\(^{2+}\) stores [22] (Fig. 3a, b). To further confirm that lysosomal Ca\(^{2+}\) did not involve in RSV-mediated TFEB activation, we examined the TFEB localization by RSV in mucolipidosis IV patient-derived TRPML1 KO (ML-IV) fibroblasts [13]. TRPML1/MCOLN1 is the principle Ca\(^{2+}\)-permeant channel on the lysosomal membrane [23]. Hence, as shown in Fig. 3c, d, RSV can induce TFEB nuclear translocation in both WT and ML-IV cells, suggesting that lysosomal Ca\(^{2+}\) is not required in RSV-mediated TFEB activation. Torin 1, a potent inhibitor of mTOR that is commonly used to induce autophagy, was used as a control. Taken together, these results suggest that the Ca\(^{2+}\) signaling induced by RSV is from ER, but not from lysosome.
We also measured the direct Ca\textsuperscript{2+} efflux by RSV with Ca\textsuperscript{2+} imaging. Acute application of RSV significantly increased cytosolic Ca\textsuperscript{2+} release in HeLa cells and this effect was dramatically reduced by a 30 min pretreatment of TG (Fig. 3e, f), but not by GPN pretreatment (Fig. 3g, h).

**Protein phosphatase 2A but not calcineurin mediates RSV-dependent TFEB dephosphorylation**

A key mechanism of TFEB activation is Ca\textsuperscript{2+}-dependent dephosphorylation of TFEB by protein phosphatases including calcineurin and PPP2/PP2A [11, 24]. We next investigated the specific phosphatase involved in RSV-mediated TFEB dephosphorylation. First, inhibition calcineurin with FK-506, a potent immunosuppressive drug commonly used to inhibit calcineurin activity, was not sufficient to block RSV-induced TFEB nuclear translocation (Fig. 4a, b). In contrast, treatment with okadaic acid (OA), a PP2A inhibitor, blocked RSV-induced TFEB nuclear translocation (Fig. 4a, b). Furthermore, following 3-6 h exposure to RSV, we found that pS211-TFEB expression levels were significantly decreased (Fig. 4c, d), indicating that TFEB was dephosphorylated at S211 by RSV, while this increase was reduced by OA (50 nM, 1 h) pretreatment (Fig. 4e, f). Consistently, RSV treatment led to TFEB band mobility shift (Fig. 4c), suggesting a post-translational modification of TFEB by RSV. Collectively, these results demonstrate that PP2A-mediated TFEB dephosphorylation underlies RSV-induced TFEB activation.

**RSV promotes autophagic flux and lysosomal function**

Our data show that RSV activates TFEB, a master regulator of autophagic and lysosomal biogenesis and function [6]. Hence, we next investigated the effect of RSV on autophagic and lysosomal function. We studied the effect of RSV on autophagy in HeLa cells stably expressing the mRFP-GFP-LC3 reporter, in which mRFP\textsuperscript{+}GFP\textsuperscript{+} and mRFP\textsuperscript{+}GFP\textsuperscript{-} puncta indicate non-acidified autophagosomes and acidified autolysosomes, respectively [25]. RSV treatment (300 µM) caused a 2.5-fold increase of the percentage of mRFP\textsuperscript{+}GFP\textsuperscript{+} yellow puncta (autophagosomes) and abundant mRFP\textsuperscript{+}GFP\textsuperscript{-} red puncta (autolysosomes), while this autophagosome increase was inhibited by BAPTA-AM pretreatment (Fig. 5a, b), suggesting
that RSV induces autophagosome formation and Ca\(^{2+}\) is required in this process. Consistently, western blot analyses revealed that RSV (300 µM, 3-9 h) treatment significantly increased LC3-II levels (Fig. 5c, d) and reduced sequestosome 1 (SQSTM1/p62) levels, a marker of autophagy flux (Fig. 5e, f). In addition, pretreatment of HeLa cells with bafilomycin A1 (BAF-A1), a V-ATPase inhibitor that can effectively block autophagosome-lysosome fusion [26], further boosted the LC3-II levels induced by RSV (300 µM, 9 h) (Fig. 5g, h). Collectively, these data suggested that RSV promotes autophagic flux.

We then explored the effects of RSV on lysosomal function. Lysosomal associated membrane protein 1 (LAMP1) is a marker of late endosomes and lysosomes (referred to as “lysosomes” for simplicity hereafter). Western blot analyses showed gradual increases in the expression of LAMP1 proteins upon RSV (300 µM, 3-9 h) treatment in a time dependent manner in HeLa cells (Fig. 6a, b). Consistently, RSV (300 µM, 6 h) significantly increased the immunofluorescence intensity of LAMP1 in HeLa cells (Fig. 6c, d). Lysosomal enzymes operate better under acidic conditions, and the degradation-active lysosomes can be tracked using LysoTracker, a fluorescent acidotropic probe [27]. Significant increases of LysoTracker staining were observed in HeLa cells following treatment with RSV (300 µM, 3-9 h) (Fig. 6e, f). Collectively, these results suggest that RSV also promotes lysosomal function and biogenesis.

**TFEB is required in RSV- mediated lipid clearance**

RSV reportedly attenuates lipid accumulation in various conditions [28], but the underlying mechanisms have not been fully clarified. We hypothesize that TFEB plays a role in RSV-mediated lipid clearance. To test this hypothesis, stearic acid (SA), a lipotoxicity inducer [29], was used to induce lipid accumulation in WT and TFEB knockout (KO) HeLa cells (Fig. 7a). In HeLa cells, SA (100 µM, 16 h) caused a nine-fold increase of lipid accumulation, and this increase was dramatically reduced by RSV (300 µM, 12 h) treatment (Fig. 7b, c). In contrast, in TFEB-KO cells, generated by using the CRISPR-Cas9 system [13], RSV-mediated lipid clearance was significantly inhibited (Fig. 7b, c). Notably, in TFEB KO cells, SA-induced the basal lipid levels were also reduced (Fig. 7b, c). In addition, pretreatment of HeLa cells with OA (50 nM, 1 h) can inhibit RSV-mediated lipid clearance.
Discussion

In this study we identify RSV, a natural polyphenol compound, induces a calcium signaling mechanism that originates from the ER and activates phosphatase PP2A to dephosphorylate TFEB. Activated TFEB then regulates the expression of genes that are required for lysosomal biogenesis and autophagosome formation, promoting lysosomal function (Fig. 8). Moreover, genetic inhibition of TFEB ameliorates the lipid clearance function of RSV, suggesting that TFEB plays an essential role in RSV-mediated lipid clearance. Collectively, our study has providing the underlying mechanism of RSV to promote lysosomal function and proposing RSV as a therapeutic candidate to treat or prevent diseases in which lysosome dysfunction has been implicated.

RSV was first discovered from the root of Veratrum nigrum L in 1940 and later found in grapes, mulberry plants, and peanuts [30]. RSV contains a spectrum of biologic activities, including immune regulation, anti-inflammation, antioxidation, and antiangiogenesis [31, 32]. Due to its multiple functions and low cytotoxicity, RSV has been approved by food and drug administration (FDA) as a dietary supplement, and commonly used in food, health care products and cosmetics [33]. Cell and animal studies show that RSV effectively reduces cellular lipid accumulation and plasma lipid levels [34, 35]. Treatment with RSV reportedly improves liver diseases including non-alcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH). Several mechanisms have been proposed for RSV including restoration of AMP-activated protein kinase (AMPK) pathway, reduction of oxidative stress and regulation of Sirt1 [36-38]. Recently dysfunctional lysosomes have been associated with the development and progression of NAFLD and NASH [39, 40]. In this study, we found that RSV can promote lysosomal function through an ER Ca$^{2+}$-activated PP2A and TFEB, which is required in RSV-mediated lipid clearance. The link we identified between RSV, TFEB, lysosomal function, and lipid clearance has revealed a unique regulation pathway.
TFEB agonists have been reported to induce Ca\(^{2+}\) release from distinct organelles, indicating Ca\(^{2+}\) homeostasis as a paramount biological modulator of TFEB activity. In this study, we show that RSV specifically triggers a Ca\(^{2+}\)-release from ER store to activate TFEB. ROS reportedly involves in several protective mechanisms of RSV [19] and can activate TFEB via a lysosomal Ca\(^{2+}\)-dependent mechanism [13]. In this study we found that ROS was not involved in RSV-mediated TFEB activation and RSV-induced TFEB activation through an ER Ca\(^{2+}\) release. This finding reveals a unique mechanism of RSV. In addition, the mechanism of different ROS induction and source of Ca\(^{2+}\) presents as an intriguing opportunity for future investigation. We also found that the ER Ca\(^{2+}\) controls the activities of phosphatase PP2A, which is consistent with previous reports that ER stress regulates PP2A activity [41]. However, the exact mechanism of this process is still unclear.

Lysosomes are the cell’s recycling center, which degrades biomolecules from endocytic and autophagic pathways [42]. In addition to the cellular degradation pathways, recent studies have revealed that lysosomes play active roles in nutrient sensing, adaption to multiple cellular stress, plasma membrane repair, cell signaling, and membrane trafficking [42]. Hence, lysosomal dysfunction has been associated to many diseases ie. lysosomal storage diseases (LSDs) [43]. As the role of lysosomes and autophagy in macromolecule metabolism becomes clearer, boosting this innate cellular clearance machinery, such as the TFEB agonists, offers a new therapeutic strategy for treatment of these diseases. In fact, pharmacological intervention of targets upstream of the TFEB, such as mTOR inhibitors and AMPK activators has been applied [44]. Our findings show that RSV can promote lysosomal function, which proposes RSV as the potential new therapeutic candidate in lysosomal dysfunction diseases.

**Methods and materials**

**Mammalian cell culture.** HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (Thermo Fisher Scientific, 11195-065) with 10% fetal bovine serum (Thermo Fisher Scientific, 10091148). All cell cultures were maintained at 37 °C in a humidified 5% CO\(_2\) incubator.
**Stable cell lines.** HeLa cells stably expressing mRFP–GFP–LC3 and GFP–TFEB were kindly provided by Drs. David Rubinsztein (University of Cambridge) and Shawn Ferguson (Yale School of Medicine), respectively as previously described [13]. HeLa TFEB-KO cells were generated and characterized as reported previously [44].

**Immunofluorescence and confocal imaging.** For immunofluorescence detection of TFEB, cells were grown and stained in coverslips in 24-well plates. 4% paraformaldehyde (Solarbio, P1110) in PBS was dispensed into each well and cells were fixed for 15 min at room temperature. 0.3% Triton X-100 was used for permeabilization for 10 min, followed by 1 h blocking with 1% bovine serum albumin (Sigma-Aldrich, WXBC2612V). The cells were incubated overnight at 4°C with anti-TFEB antibodies (Cell Signaling Technology, 4240) (1:1000). After washing for 5 times with PBS, secondary antibodies conjugated to Alexa Fluor -488 (Thermo Fisher Scientific, 1705869) (1:1000) were added and incubated for 1 h in a dark place. DAPI (Sigma-Aldrich, D9542) was added to stain nuclei for 10 min and briefly washed for 4 times. Coverslips were then mounted with Fluoromount-G (Southern Biotech) and images were acquired with an Olympus or Zeiss confocal microscope.

**Calcium imaging.** Fura-2 Ca\(^{2+}\) imaging was performed in HeLa cells loaded with 10 µM Fura-2 AM (Invitrogen) at 37 °C for 30 min, as mentioned previously. In brief, the EasyRatioPro system (PTI) was used to record fluorescence, at two excitation wavelengths, 340 nm and 380 nm. Fura-2 AM ratios (F\(_{340}/F_{380}\)) were used to monitor changes in intracellular [Ca\(^{2+}\)]. Cells were bathed in Tyrode’s solution containing 145 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM glucose, and 20 mM HEPES (pH7.4). Ca\(^{2+}\) release was measured under a zero-Ca\(^{2+}\) external solution, which contained 145 mM NaCl, 5 mM KCl, 3 mM MgCl\(_2\), 10 mM glucose, 1 mM EGTA and 20 mM HEPES (pH 7.4). RSV (in a zero Ca\(^{2+}\) solution) was used to induce Ca\(^{2+}\) release. Ionomycin (1 µM) was added at the end of all experiments to induce a maximal response for comparison. All responses were estimated with Maxchelator software.
**Western blotting.** Cells were lysed in RIPA buffer supplemented with 1x protease inhibitors cocktail (Sigma-Aldrich, P8340) (1:100) and 1x phosphatase inhibitor cocktail (Abcam, GR304037-28) on ice for 20 min. Protein samples were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels followed by transferring to polyvinylidene difluoride membranes (Merck, R7DA8778E). The membranes were blocked with 5% nonfat dry milk for 1 h and incubated with antibodies against TFEB (Cell Signaling Technology, 37785) (1:500), pS211-TFEB (Cell Signaling Technology, 37681) (1:500), LC3 (Sigma-Aldrich, L8918) (1:1000), LAMP1 (Abcam, ab24170) (1:1,000), SQSTM1 (Cell Signaling Technology, 5114) (1:1000) and GAPDH (Sigma-Aldrich, G9545) (1:10,000), respectively. Bound antibodies were detected horseradish peroxidase-conjugated anti-rabbit (Abcam, ab6721) or anti-mouse secondary antibodies (Abcam, ab6789) (1:10,000) in 5% nonfat dry milk for 1 h at room temperature with agitation. And enhanced chemiluminescence reagents (Amersham Pharmacia Biotech) was applied. Band intensities were quantified using the NIH Image J software.

**LysoTracker staining.** Lysosomal luminal pH was measured by LysoTracker Red DND-99 staining. HeLa cells were incubated with 50 nM LysoTracker Red DND-99 (Invitrogen, L7528) at 37 °C for 30 min. The LysoTracker signal was excited at 562 nm, and emission was read at 595 nm using a laser scanning confocal immunofluorescence microscope.

**Quantitative RT-PCR.** For the synthesis of cDNA, total RNA was isolated using Trizol (ThermoFisher Scientific, 191002), according to the manufacture’s protocol. cDNA was generated with GoScript Reverse Transcription System (Promega, 0000316057). Quantitative real-time PCR was performed using the SYBR Green Mix (TOYOBO, 563700) in ABI 7500 FAST (Applied Biosystems). The relative mRNA expression level of each target gene in treated cells was normalized to that measured in untreated cells: relative mRNA expression level=$2^{-\Delta\Delta CT}$ (treated cells)-$\Delta CT$ (untreated cells)]. The primers used in this study as following:

CTSD: For 5’- CTTCGACAACCTGATGCAGC-3’, Rev 5’- TACTTGAGTCTGTGCCACC-3’
Oil red O staining. Lipid accumulation was analyzed by Oil red O staining. Cells were fixed with 10% formalin for 30 min and washed with PBS three times. Cells were then incubated with 60% isopropanol for 5 min and stained with Oil Red O (Sigma-Aldrich, SLBR6841V) solution (stock solution, 0.5g/ml in isopropanol: working solution, 30 ml of Oil red O stock solution and 20 ml of distilled water) for 10 min at room temperature. Unbound dye was washed by water. Images were then taken by Nikon ECLIPSE Ti-S microscope.

Reagents
Chemicals used in this study including RSV (TargetMol, T1558), BAPTA-AM (Thermo Fisher Scientific, 1824047), NAC (Sigma-Aldrich, A7250), GPN (Santa Cruz Biotech, sc-252858), TG (Sigma-Aldrich, T9033), OA (Sigma-Aldrich, O7760), Torin1 (Cell signaling technology, 14379), FK506 (Sigma-Aldrich, F4679), BAF-A1 (ALADDIN, 88899-55-2).

Statistical analysis. Data are presented as mean ± s.e.m. from at least 3 independent experiments. Statistical comparisons were performed with analyses of variance (ANOVA) or two tailed Student’s t-test with paired or unpaired wherever appropriate. A P value < 0.05 was considered statistically significant.

Acknowledgements
This work was supported by a National Natural Science Foundation of China grant (NSFC, 31600823). The funders had no role in study design, data collection and analysis, decision to
publish, or preparation of the manuscript. We are grateful to Dr. Shawn M. Ferguson for the GFP-TFEB stable cells, to Dr. David Rubinsztein for the mRFP-GFP-LC3 cells and to Dr. Haoxing Xu for the TFEB-KO stable cells.

Disclosure statement

No potential conflict of interest was reported by the authors.

Author contributions

D. Li and R. Shao designed research; R. Shao, J. Shi, K. Du, N. Wang, W. Cai, S. Liu, Z. Ding and Y. Wang performed the laboratory experiments; R. Shao, J. Shi, K. Du, W. Cai, S. Liu and Z. Ding analysed and interpreted the data; D. Li wrote the paper with inputs and final approval from all authors.
References

1. Klionsky, D.J. and S.D. Emr, Cell biology - Autophagy as a regulated pathway of cellular degradation. Science, 2000. 290(5497): p. 1717-1721.

2. Mizushima, N. and M. Komatsu, Autophagy: Renovation of Cells and Tissues. Cell, 2011. 147(4): p. 728-741.

3. Singh, R., et al., Autophagy regulates lipid metabolism. Nature, 2009. 458(7242): p. 1131-U64.

4. Ding, W.X., et al., Autophagy Reduces Acute Ethanol-Induced Hepatotoxicity and Steatosis in Mice. Gastroenterology, 2010. 139(5): p. 1740-1752.

5. Steingrimsson, E., N.G. Copeland, and N.A. Jenkins, Melanocytes and the microphthalmia transcription factor network. Annual Review of Genetics, 2004. 38(38): p. 365-411.

6. Settembre, C., et al., TFEB links autophagy to lysosomal biogenesis. Science, 2011. 332(6036): p. 1429-33.

7. Settembre, C., et al., TFEB controls cellular lipid metabolism through a starvation-induced autoregulatory loop. Nature Cell Biology, 2013. 15(6): p. 647-+.  

8. Settembre, C., et al., A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. EMBO J, 2012. 31(5): p. 1095-108.

9. Ferron, M., et al., A RANKL-PKCbeta-TFEB signaling cascade is necessary for lysosomal biogenesis in osteoclasts. Genes Dev, 2013. 27(8): p. 955-69.

10. Martina, J.A. and R. Puertollano, RRAG GTPases link nutrient availability to gene expression, autophagy and lysosomal biogenesis. Autophagy, 2013. 9(6): p. 928-30.

11. Medina, D.L., et al., Lysosomal calcium signalling regulates autophagy through calcineurin and TFEB. Nat Cell Biol, 2015. 17(3): p. 288-99.

12. Roczniakferguson, A., et al., The Transcription Factor TFEB Links mTORC1 Signaling to Transcriptional Control of Lysosome Homeostasis. Science Signaling, 2012. 5(228): p. ra42.

13. Zhang, X., et al., MCOLN1 is a ROS sensor in lysosomes that regulates autophagy. Nat Commun, 2016. 7: p. 12109.

14. Lapierre, L.R., et al., The TFEB orthologue HLH-30 regulates autophagy and modulates longevity in Caenorhabditis elegans. Nat Commun, 2013. 4: p. 2267.

15. Sha, L., et al., Targeting Mixed Lineage Leukemia (MLL): Drug Discovery from Natural Product Extracts (NPEs). American Journal of Pathology, 2018. 188(10): p. 2419-2419.

16. Vicinanza, M., et al., PI(5)P regulates autophagosome biogenesis. Mol Cell, 2015. 57(2): p. 219-34.

17. Visalli, G., et al., In vitro assessment of the indirect antioxidant activity of Sulforaphane in redox imbalance vanadium-induced. Nat Prod Res, 2017. 31(22): p. 2612-2620.

18. Zhou, X., et al., Resveratrol attenuates endothelial oxidative injury by inducing autophagy via the activation of transcription factor EB. Nutr Metab (Lond), 2019. 16: p. 42.
19. Li, Q., et al., *Resveratrol Sensitizes Carfilzomib-Induced Apoptosis via Promoting Oxidative Stress in Multiple Myeloma Cells*. Front Pharmacol, 2018. 9: p. 334.

20. Clapham, D.E., *Calcium signaling*. Cell, 2007. 131(6): p. 1047-58.

21. Lytton, J., M. Westlin, and M.R. Hanley, *Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps*. J Biol Chem, 1991. 266(26): p. 17067-71.

22. Berg, T.O., et al., *Use of glycy1-L-phenylalanine 2-naphthylamide, a lysosome-disrupting cathepsin C substrate, to distinguish between lysosomes and prelysosomal endocytic vacuoles*. Biochem J, 1994. 300 ( Pt 1): p. 229-36.

23. Dong, X.P., et al., *The type IV mucolipidosis-associated protein TRPML1 is an endolysosomal iron release channel*. Nature, 2008. 455(7215): p. 992-6.

24. Martina, J.A. and R. Puertollano, *Protein phosphatase 2A stimulates activation of TFEB and TFE3 transcription factors in response to oxidative stress*. J Biol Chem, 2018. 293(32): p. 12525-12534.

25. Vicinanza, M.K., Viktor?I., Ashkenazi, Avraham, *PI(5)P regulates autophagosome biogenesis*. Molecular Cell, 2015. 57(2): p. 219-234.

26. Yoshimori, T., et al., *Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells*. J Biol Chem, 1991. 266(26): p. 17707-12.

27. Li, P., M. Gu, and H. Xu, *Lysosomal Ion Channels as Decoders of Cellular Signals*. Trends Biochem Sci, 2019. 44(2): p. 110-124.

28. Huang, Y., et al., *Resveratrol protects against nonalcoholic fatty liver disease by improving lipid metabolism and redox homeostasis via the PPARalpha pathway*. Appl Physiol Nutr Metab, 2019.

29. Spigoni, V., et al., *Stearic acid at physiologic concentrations induces in vitro lipotoxicity in circulating angiogenic cells*. Atherosclerosis; p. S0021915017312716.

30. Bertelli, A.A.E., *Modulatory effect of resveratrol, a natural phytoalexin, on endothelial adhesion molecules and intracellular signal transduction*. Pharmaceutical Biology, 1998.

31. Bellaver, B., et al., *Resveratrol increases antioxidant defenses and decreases proinflammatory cytokines in hippocampal astrocyte cultures from newborn, adult and aged Wistar rats*. Toxicol In Vitro, 2014. 28(4): p. 479-84.

32. Fan, E., et al., *Beneficial effects of resveratrol on atherosclerosis*. J Med Food, 2008. 11(4): p. 610-4.

33. Park, S.J., et al., *Resveratrol ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases*. Cell, 2012. 148(3): p. 421-33.

34. Li, Y.T., et al., *Resveratrol ameliorates lipid accumulation in HepG2 cells associated with down-regulation of lipin1 expression*. Revue Canadienne De Physiologie Et Pharmacologie, 2016. 94(2): p. 1.

35. Haghighatdoost, F. and M. Hariri, *Effect of resveratrol on lipid profile: An updated systematic review and meta-analysis on randomized clinical trials*. Pharmacol Res, 2018. 129: p. 141-150.
36. Shang, J., et al., Resveratrol improves non-alcoholic fatty liver disease by activating AMP-activated protein kinase. Acta Pharmacol Sin, 2008. 29(6): p. 698-706.

37. Arcanjo, N.O., et al., Resveratrol protects Lactobacillus reuteri against H2O2-induced oxidative stress and stimulates antioxidant defenses through upregulation of the dhaT gene. Free Radic Biol Med, 2019. 135: p. 38-45.

38. Zhou, R., et al., Resveratrol Ameliorates Lipid Droplet Accumulation in Liver Through a SIRT1/ATF6-Dependent Mechanism. Cell Physiol Biochem, 2018. 51(5): p. 2397-2420.

39. Du, J., et al., Cellular endo-lysosomal dysfunction in the pathogenesis of non-alcoholic fatty liver disease. Liver Int, 2020. 40(2): p. 271-280.

40. Liu, W., et al., Macrophage Raptor Deficiency-Induced Lysosome Dysfunction Exacerbates Nonalcoholic Steatohepatitis. Cell Mol Gastroenterol Hepatol, 2019. 7(1): p. 211-231.

41. He, C., et al., ER stress regulating protein phosphatase 2A-B56gamma, targeted by hepatitis B virus X protein, induces cell cycle arrest and apoptosis of hepatocytes. Cell Death Dis, 2018. 9(7): p. 762.

42. Xu, H. and D. Ren, Lysosomal physiology. Annu Rev Physiol, 2015. 77: p. 57-80.

43. Luzio, J.P., P.R. Pryor, and N.A. Bright, Lysosomes: fusion and function. Nat Rev Mol Cell Biol, 2007. 8(8): p. 622-32.

44. !!! INVALID CITATION !!!
Figure Legends

**Figure 1.** RSV activates TFEB and expression of TFEB downstream genes. (a) Chemical structure of RSV. (b) Effects of RSV on TFEB nuclear translocation in HeLa GFP-TFEB stable cells. Cells were treated with RSV (100-300 µM) for 4 h. Nuclei were counterstained with DAPI (blue). Scale bar, 10 µm. (c) Ratio of nuclear versus cytosolic TFEB shown in b. (N = 30 cells randomly-selected cells from 3 independent experiments). (d) RSV (300 µM, 4 h) induced endogenous TFEB nuclear translocation in HeLa cells. Nuclei were counterstained with DAPI (blue). Scale bar, 10 µm. (e) Quantification of results shown in d. (N = 30 cells from 3 independent experiments). (f) RSV (300 µM, 12 h) upregulated the mRNA expression of TFEB downstream genes (CTSD, NEU1, SQSTM1, WIP1, LAMP1) and antioxidant related genes (HO1, NQO1) in HeLa cells analyzed by QPCR (n=3 independent experiments). For all panels, data are presented as mean ±s.e.m.; **p < 0.01, ***p < 0.001, ANOVA.

**Figure 2.** Chelation of intracellular Ca^{2+} suppresses RSV-induced TFEB nuclear translocation. (a) RSV (300 µM, 4 h)-induced GFP-TFEB nuclear translocation was blocked by BAPTA-AM (10 µM, 1 h) pretreatment in HeLa GFP-TFEB stable cells. Nuclei were counterstained with DAPI. Scale bar, 10 µm. (b) Average ratios of nuclear vs. cytosolic TFEB shown in a. (N=30-40 randomly-selected cells from 4 independent experiments). (c) BAPTA-AM (10 µM, 1 h) pretreatment blocked RSV-induced endogenous TFEB nuclear translocation in HeLa WT cells. (d) Quantification of results shown in c. (N = 30 cells from 3 independent experiments). (e) The effect of NAC on RSV-induced TFEB translocation. HeLa GPF-TFEB cells were co-treated with NAC (5 mM) for 4 h. Scale bar, 10 µm. (f) Quantification of results shown in e. (N = 30 cells from 3 independent experiments). For all panels, data are presented as mean ±s.e.m.; **p < 0.01, ***p < 0.001, ANOVA.

**Figure 3.** ER source of Ca^{2+} -dependence of RSV-induced TFEB activation. (a) In HeLa cells stably expressing GFP-TFEB, pretreatment with 300 nM TG for 30 min inhibited RSV-induced TFEB nuclear translocation. Pretreatment with 200 µM GPN had no effect. Scale bar, 10 µm. (b) The graph represents the average ratios of TFEB in nuclear versus cytosolic under conditions in a. (N = 40 cells from 3 independent experiments). (c) RSV (300 µM, 4 h) induced endogenous TFEB nuclear translocation in HeLa cells. Nuclei were counterstained with DAPI (blue). Scale bar, 10 µm. (d) Quantification of results shown in c. (N = 30 cells from 3 independent experiments). For all panels, data are presented as mean ±s.e.m.; **p < 0.01, ***p < 0.001, ANOVA.
µM, 4 h) induced TFEB nuclear translocation in both WT and ML-IV (TRPML1-null) human fibroblast cells. Torin 1 (1 µM) was used as a positive control to induce autophagy. Scale bar, 10 µm. (d) Quantification of results shown in c. (N = 40 cells randomly selected from 3 independent experiments). (e) Bath application of RSV (300 µM) resulted in Ca$^{2+}$ release in HeLa cells, measured with Ca$^{2+}$-sensitive dye Fura-2 ($F_{340}/F_{380}$). TG (300 nM, 30 min) treatment abolished RSV-induced Ca$^{2+}$ release. (f) Quantification of results shown in e. from N=30 cells randomly selected from three representative experiments. (g) Pretreatment with GPN (200 µM, 30 min) had no effect on the response to RSV-induced Ca$^{2+}$ release in HeLa cells. (h) Quantification of GPN pretreatment responses shown in g. (N=20–30 cells from three representative experiments). Data are presented as mean ± s.e.m., ***p < 0.001, ANOVA.

**Figure 4.** Phosphatase PP2A mediates RSV-induced dephosphorylation of TFEB. (a) Effects of FK506 (5 µM) or OA (50 nM) pretreatment for 1 h on RSV-induced TFEB activation in HeLa GFP-TFEB stable cells. Scale bar, 10 µm. (b) Quantitative analysis of TFEB location as shown in a. (N = 30 cells randomly-selected cells from 3 independent experiments). (c) Western blot analysis of TFEB phosphorylation, assayed by ratios of pS211-TFEB vs. total TFEB in RSV (300 µM, 3-6 h) -treated HeLa cells. Experiments were repeated multiple times and a representative blot was shown. (d) Quantification of the results shown in c. from n = 3 independent experiments. (e) Western blot analysis of ratios of pS211-TFEB vs. total TFEB with or without RSV (300 µM, 6 h) treatment and in the presence and absence of OA (50 nM, 1 h) pretreatment in HeLa cells. (f) Quantification of the results shown in e. from n = 3 independent experiments. For all panels, data are presented as mean ± s.e.m., ***p < 0.001, ANOVA.

**Figure 5.** RSV promotes autophagic flux. (a) In HeLa cells stably expressing mRFP-GFP-LC3, RSV treatment (300 µM, 9 h) increased the formation of autophagosomes, visualized as mRFP$^+$ GFP$^+$ yellow puncta and this increase was inhibited by BAPTA-AM pretreatment (10 µM, 1 h). Scale bar, 10 µm or 2 µm (for zoom-in images). (b) The graph represents the percentage of yellow puncta shown in a. (N = 30 cells randomly-selected cells
from 3 independent experiments). (c) Western blot analysis of LC3-II protein expression in RSV (300 µM) -treated HeLa cells for indicated time. (d) Quantitative analysis of relative LC3-II protein levels shown in c. (n= 3). (e) Western blot analysis of SQSTM1/p62 and LC3-II protein expression level with RSV (300 µM, 9 h) treatment in HeLa cells. (f) Quantitative analysis of the results shown in e. (n= 3 independent experiments). (g) Western blot analysis of LC3-II protein expression level with or without RSV (300 µM, 9 h) treatment, and in the presence and absence of Baf-A1 (0.5 µM), used as an inhibitor of autophagosome-lysosome fusion in HeLa cells. Torin 1 (1 µM) was used as a positive control to induce autophagy. (h) Quantitative analysis of LC3-II levels under various experimental conditions shows in e. (n= 3 independent experiments). For all panels, data are presented as mean ±s.e.m., ***p < 0.001, ANOVA.

Figure 6. RSV promotes lysosome function. (a) Western blot analysis of LAMP1 protein expression in HeLa cells treated with RSV (300 µM) for indicated time. (b) Quantification of results shown in a. (n=3 independent experiments). (c) LAMP1 immunofluorescence staining in RSV (300 µM, 6 h) treated HeLa cells. Scale bar, 10 µm or 2 µm (for zoom-in images). (d) Quantitative of LAMP1 fluorescence intensity shown in c. (N = 30 cells randomly-selected cells from 3 independent experiments). (e) Effects of RSV on lysosome acidification. HeLa cells were incubated with 300 µM RSV for 3-9 h and then stained with LysoTracker (50 nM, 15 min) to label acidified lysosomes. Scale bar, 10 µm or 2 µm (for zoom-in images). (f) Quantitative analysis of LysoTracker intensity as shows in e. (N = 30 randomly-selected cells from 3 independent experiments). For all panels, data are presented as mean ±s.e.m., ***p < 0.001, ANOVA.

Figure 7. TFEB is required in RSV-mediated lipid accumulation. (a) Western blot analysis of TFEB protein expression in WT and TFEB-KO HeLa cells. GAPDH was used as an internal control. (b) Oil Red O analysis of lipid levels in RSV (300 µM, 12 h)-treated WT and TFEB-KO HeLa cells with pretreatment with SA (100 µM, 16 h). Scale bar, 10µm. (c) Quantitative analysis of results shown in b. (n=3 independent experiments). (d) Effects of OA pretreatment (50 nM, 1 h) on RSV (300 µM, 12 h) treated HeLa cells with pretreatment
with SA (100 μM, 16 h). Scale bar, 10μm. (e) Quantitative analysis of results shown in d. (n=3 independent experiments). For all panels, data are presented as mean ± s.e.m., ***p < 0.001, ANOVA.

**Figure 8.** A working model to illustrate the role of TFEB in RSV-mediated lipid clearance. RSV stimulates a Ca\(^{2+}\) signaling released from ER, which activates phosphatase PP2A to dephosphorylate TFEB. Activated TFEB then translocates from cytoplasm to nucleus, and binds to CLEAR elements, promoting the transcription expression of autophagic and lysosomal genes. Subsequently, promoted lysosomal biogenesis and autolysosome formation accelerates the lipid clearance.
Figure 1

a) Structure of Resveratrol (RSV)

b) HeLa TFEB stable

c) CTL 100 300

HeLa TFEB stable

RSV (100 μM)

RSV (300 μM)

d) HeLa

CTT 100 300

HeLa

HeLa

anti-TFEB DAPI Merge

GFP-TFEB DAPI Merge

Merge

e) HeLa

CTT 100 300

HeLa

HeLa

anti-TFEB DAPI Merge

GFP-TFEB DAPI Merge

Merge

f) DMSO RSV

CTSD NEU1 SOSTM1 WIPI LAMP1 HO1 NQO1

Fold enrichment

*** *** *** *** *** ***
Figure 2
Figure 3

(a) HeLa TFEB stable

CTL

GFP-TFEB, DAPI, Merge

GPN

GFP-TFEB, DAPI, Merge

TG

GFP-TFEB, DAPI, Merge

DMSO, RSV

(b) CTL, GPN, TG

TFEB nuc/cyt ratio

DMSO, RSV

(c) WT, MLIV

anti-TFEB, DAPI, Merge

DMSO, RSV, Torin1

(d) CTL, RSV, Torin1

TFEB nuc/cyt ratio

WT, MLIV

(e) HeLa

0 Ca\(^{2+}\), RSV, Tyrode's

Fura-2(F340/F380)

Time(s)

(f) HeLa

0 Ca\(^{2+}\), RSV, Tyrode's

Fura-2(F340/F380)

Time(s)

(g) HeLa

0 Ca\(^{2+}\), RSV, Tyrode's

Fura-2(F340/F380)

Time(s)

(h) HeLa

0 Ca\(^{2+}\), RSV, Tyrode's

Fura-2(F340/F380)

Time(s)
Figure 4

(a) CTL FK506 OA

|               | DMSO | RSV |
|---------------|------|-----|
| GFP-TFEB      |      |     |
| DAPI          |      |     |
| Merge         |      |     |
| GFP-TFEB      |      |     |
| DAPI          |      |     |
| Merge         |      |     |

(b) DMSO □ RSV

![Graph showing TFEB nuc/cyt ratio](image)

(c) HeLa

|            | 0   | 3   | 6   |
|------------|-----|-----|-----|
| RSV (h)    |     |     |     |
| pS211-TFEB | ![Image](image) |
| TFEB       | ![Image](image) |
| GAPDH      | ![Image](image) |

(d) Normalized pS211-TFEB: total TFEB levels

![Graph showing normalized pS211-TFEB](image)

(e) HeLa

|            | OA  | RSV |
|------------|-----|-----|
|            |     |     |     |
| pS211-TFEB | ![Image](image) |
| TFEB       | ![Image](image) |
| GAPDH      | ![Image](image) |

(f) Normalized pS211-TFEB: total TFEB levels

![Graph showing normalized pS211-TFEB](image)
**Figure 5**
Figure 6
Figure 7

(a) HeLa WT TFEB-KO
TFEB
GAPDH

(b) HeLa WT TFEB-KO

(c) Oil Red O (%Area)

WT
TFEB-KO

*** *** N.S.

DMSO SA SA+RSV

(c) GAPDH TFEB

30 60
HeLa

(d) HeLa

CTL OA

DMSO SA SA+RSV

(e) Oil-Red O (%Area)

DMSO SA SA+RSV

*** *** N.S.

DMSO SA SA+RSV

CTL OA

Downloaded from http://portlandpress.com/biochemj/article-pdf/doi/10.1042/BCJ20200676/904500/bcj-2020-0676.pdf by guest on 20 February 2021
Figure 8