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Lysosomal Ca\textsuperscript{2+}-mediated TFEB activation modulates mitophagy and functional adaptation of pancreatic β-cells to metabolic stress

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Although autophagy is critical for pancreatic β-cell function, the role and mechanism of mitophagy in β-cells are unclear. We studied the role of lysosomal Ca\textsuperscript{2+} in TFEB activation by mitochondrial or metabolic stress and that of TFEB-mediated mitophagy in β-cell function. Mitochondrial or metabolic stress induced mitophagy through lysosomal Ca\textsuperscript{2+} release, increased cytosolic Ca\textsuperscript{2+} and TFEB activation. Lysosomal Ca\textsuperscript{2+} replenishment by ER>lysosome Ca\textsuperscript{2+} refilling was essential for mitophagy. β-cell-specific Tfeb knockout (Tfeb\textsuperscript{Δβ}-cell) abrogated high-fat diet (HFD)-induced mitophagy, accompanied by increased ROS and reduced mitochondrial cytochrome c oxidase activity or O\textsubscript{2} consumption. Tfeb\textsuperscript{Δβ}-cell mice showed aggravation of HFD-induced glucose intolerance and impaired insulin release. Metabolic or mitochondrial stress induced TFEB-dependent expression of mitophagy receptors including Ndp52 and Optn, contributing to the increased mitophagy. These results suggest crucial roles of lysosomal Ca\textsuperscript{2+} release coupled with ER>lysosome Ca\textsuperscript{2+} refilling and TFEB activation in mitophagy and maintenance of pancreatic β-cell function during metabolic stress.
Mitochondrial function is essential for insulin release and survival of pancreatic β-cells. Mitochondrial function is maintained by several factors such as mitochondrial biogenesis, fission, fusion, and selective autophagy (mitophagy). The importance of autophagy in the maintenance of β-cell function and mitochondria has been demonstrated in previous studies using β-cell-specific autophagy knockout (KO) (Alg2ΔP-cell) mice which had reduced β-cell mass and mitochondrial abnormalities. However, the role and mechanism of mitophagy in pancreatic β-cells have not been clearly demonstrated.

Transcription factor EB (TFEB) acts as a master regulator of lysosome biogenesis and autophagy gene expression. TFEB family members also play a role in mitophagy. In TFEB activation and mitophagy by mitochondrial stressors, lysosomal Ca²⁺ release plays an important part. However, the mechanism that perpetuates lysosomal Ca²⁺ release and enables the full progression of mitophagy despite the small lysosomal volume is not clear.

We investigated the mechanism and functional role of mitophagy in pancreatic β-cells and found that TFEB is activated by mitochondrial reactive oxygen species (ROS)-induced lysosomal Ca²⁺ release through calcineurin. We also obtained data indicating the importance of ER→lysosome Ca²⁺ refilling in the sustained release of lysosomal Ca²⁺. TFEB activation and mitophagy by mitochondrial stressors. Finally, we observed that TFEB contributes to β-cell mitophagy through induction of mitophagy receptors such as Calcoco2 (Ndp52) and Optn.

Results
Mitophagy and TFEB nuclear translocation by mitochondrial stressors. We first studied whether mitophagy occurs in INS-1 insulinoma cells treated with mitochondrial stressors. Immunoblotting showed that INS-1 cells express Parkin (Supplementary Fig. 1a), which is necessary for mitophagy by mitochondrial stressors. When we transfected cells with pMito-Keima encoding a pH-sensitive fluorescent probe with a mitochondria-targeting sequence, tubular mitochondrial Keima fluorescence excited by 440-nm laser was observed. After rotenone treatment, a mitochondrial complex I inhibitor, many cells showed punctate fluorescence excited by a 590-nm laser (acidic puncta), indicating lysosomal delivery of mitochondrial Keima and mitophagy (Fig. 1a). When cells were treated with another mitochondrial stressor antimycin A, a mitochondrial complex III inhibitor, combined with oligomycin, an ATP synthase inhibitor (O/A), acidic Mito-Keima puncta were again well observed, indicating mitophagy (Fig. 1a). To confirm mitophagy, cells were transfected with mRFP-LC3 and treated with rotenone or O/A. In many cells, mRFP-LC3 puncta colocalized with TOM20, a mitochondrial outer membrane protein, were observed by immunofluorescence (Fig. 1b), supporting rotenone- or O/A-induced mitophagy in INS-1 cells.

We next studied the role of TFEB, a master regulator of lysosomal biogenesis and autophagy genes, in mitophagy because transcriptional control of autophagy/lysosomal genes could be important in mitophagy. When we studied TFEB nuclear translocation, a crucial event in TFEB activation, using TFEB-GFP-transfected INS-1 cells, TFEB nuclear fluorescence was apparent after rotenone or O/A treatment (Fig. 1c), similar to previous results using HeLa cells. When INS-1 cells transfected with GFP conjugated to TF3E, another member of the MITF/TFE family (TFE3-GFP), TF3E nuclear fluorescence was clearly observed after rotenone or O/A treatment as well (Fig. 1c). Nuclear translocation of endogenous TFEB and TF3E was also observed after rotenone or O/A treatment (Fig. 1d). To study the role of TFEB/TF3E activation in mitophagy, the role of TFEB/TF3E activation in mitophagy, the role of TFEB/TF3E activation in mitophagy, we used BAPTA-AM, a cell-permeable Ca²⁺ chelator. BAPTA-AM pretreatment significantly reduced the proportion of cells with nuclear TFEB and the number of acidic Mito-Keima puncta by rotenone or O/A (Fig. 2i and Supplementary Fig. 3a), indicating TFEB nuclear translocation and mitophagy by cytoplasmic Ca²⁺ released from lysosome.

Since increased [Ca²⁺], by rotenone or O/A induced TFEB nuclear translocation and mitophagy through calcineurin, a phosphatase, we next studied changes in TFEB dephosphorylation by rotenone or O/A. After treatment with rotenone or O/A, calcineurin activity was markedly increased, suggesting TFEB dephosphorylation (Supplementary Fig. 4a). Consistently, phosphorylation at S142, one of the most important

Lysosomal Ca²⁺ in mitophagy. To study the mechanism of TFEB nuclear translocation by mitochondrial stressors, we examined whether mitochondrial stressor-induced mitophagy involves calcineurin, one of the most important phosphatases regulating TFEB activation in starvation. When INS-1 cells transfected with TFEB-GFP and HA-ΔCna-H151Q, a dominant-negative mutant of calcineurin, were treated with rotenone or O/A, TFEB nuclear translocation by mitochondrial stressors was significantly reduced (Fig. 2a, b), indicating the role of calcineurin in TFEB activation by mitochondrial stressors. We also studied the role of calcineurin in mitophagy. In cells transfected with mRFP-LC3 and HA-ΔCna-H151Q and then treated with rotenone or O/A, mitophagy identified by mRFP-LC3 puncta colocalized with TOM20, was also significantly reduced (Fig. 2c, d), showing the role of calcineurin in mitophagy by mitochondrial stressors.
TFEB phosphorylation sites\(^3\), was markedly reduced by rotenone or O/A (Supplementary Fig. 4a). We also studied phosphorylation at S211 of TFEB, another important phosphorylation site, using immunoprecipitation assay based on TFEB pS211 binding to 14-3-3 protein\(^5\). In immunoblotting, band intensity of 14-3-3 binding motif of TFEB, thus pS211-TFEB level\(^5\), in TFEB immunoprecipitate was markedly reduced by rotenone or O/A treatment of TFEB-GFP-transfectants (Supplementary Fig. 4b). Similarly, band intensity of 14-3-3 binding motif of TFE3, thus pS321-TFE3 level\(^6\), in TFE3 immunoprecipitate was markedly reduced by rotenone or O/A treatment of TFE3-GFP-transfectants (Supplementary Fig. 4b). In addition, we reproducibly observed reduced expression of total TFEB or TFE3 after O/A treatment (Supplementary Fig. 4b), suggesting possible degradation of TFEB or TFE3, while the mechanism and significance of these results are unknown.

We next studied the role of TRPML1 channel, a lysosomal Ca\(^{2+}\) channel that can be crucial in autophagy\(^7\). ML-Si3, a
TRPML1 channel antagonist\(^{18}\), significantly reduced \([\text{Ca}^{2+}]_i\) increase by rotenone or O/A (Supplementary Fig. 3b), suggesting \([\text{Ca}^{2+}]_i\) release by mitochondrial stressors through TRPML1. MLO-SI3 also significantly reduced mitophagy by rotenone or O/A (Supplementary Fig. 3a). To obtain genetic evidence, we conducted knockdown (KD) of Mcoln1 encoding TRPML1. RT-PCR confirmed markedly reduced Mcoln1 expression by siMcoln1 transfection (Supplementary Fig. 3c). In Mcoln1-KD cells, rotenone- or O/A-induced mitophagy detected as acidic Mito-Keima puncta was significantly reduced (Supplementary Fig. 3d), demonstrating a crucial role of TRPML1 in mitochondrial stressor-induced mitophagy.

We next studied the mechanism of lysosomal \([\text{Ca}^{2+}]_i\) release through TRPML1 by mitochondrial stressors. We examined ROS accumulation because ROS affects diverse ion channels, and can be generated by mitochondrial stressors in the vicinity of lysosome\(^{19}\). We observed strong ROS accumulation stained with CM-H2DCFDA by rotenone or O/A (Supplementary Fig. 5a). Pretreatment with N-acetylcysteine (NAC), an antioxidant, markedly reduced ROS accumulation by rotenone or O/A (Supplementary Fig. 5a). Intriguingly, NAC significantly inhibited rotenone- or O/A-mediated increase of \([\text{Ca}^{2+}]_i\) (Supplementary Fig. 5b), which suggests that ROS induces lysosomal \([\text{Ca}^{2+}]_i\) release and increases \([\text{Ca}^{2+}]_i\), leading to TEBF nuclear translocation. We also studied changes in mitochondrial ROS that can be more relevant to the induction of mitophagy, using MitoSOX. Again, mitochondrial ROS was increased by rotenone or O/A, which was significantly reduced by MitoTEMPO, a mitochondrial ROS scavenger (Supplementary Fig. 5c). Furthermore, both the increase of \([\text{Ca}^{2+}]_i\) and the decrease of \([\text{Ca}^{2+}]_i\) by rotenone or O/A were suppressed by MitoTEMPO (Supplementary Fig. 5d, e), indicating the role of mitochondrial ROS in lysosomal \([\text{Ca}^{2+}]_i\) efflux, increased \([\text{Ca}^{2+}]_i\), and decreased \([\text{Ca}^{2+}]_i\), by mitochondrial stressors.

**Role of ER \(\rightarrow\) lysosome \([\text{Ca}^{2+}]_i\) refling in mitophagy.** Although \([\text{Ca}^{2+}]_i\) is comparable to \([\text{Ca}^{2+}]_i\) in ER, lysosome is a minor \([\text{Ca}^{2+}]_i\) reservoir because of a small lysosomal volume and might not be a sufficient source to ensure full progression of intracellular events requiring \([\text{Ca}^{2+}]_i\). Therefore, we studied whether ER \(\rightarrow\) lysosome \([\text{Ca}^{2+}]_i\) movement that can follow lysosomal \([\text{Ca}^{2+}]_i\) depletion\(^{20}\), occurs during mitophagy. We observed that decreased \([\text{Ca}^{2+}]_i\) by rotenone or O/A recovered after mitochondrial stressor removal (Fig. 3a). Since ER, the largest \([\text{Ca}^{2+}]_i\) reservoir in cells, is the most likely source replenishing depleted lysosomal \([\text{Ca}^{2+}]_i\) store\(^\text{6}\), we conducted blockade of ER \([\text{Ca}^{2+}]_i\) exit channels that could be routes for ER \(\rightarrow\) lysosome \([\text{Ca}^{2+}]_i\) movement. Xestospongin C, an inositol trisphosphate receptor (InsP3R) antagonist, significantly suppressed \([\text{Ca}^{2+}]_i\) recovery after mitochondrial stressor removal (Fig. 3a), suggesting the occurrence of ER \(\rightarrow\) lysosome \([\text{Ca}^{2+}]_i\) refling during mitophagy through InsP3R. Dantrolene, an antagonist of ryadine receptor, another ER \([\text{Ca}^{2+}]_i\) exit channel, similarly inhibited \([\text{Ca}^{2+}]_i\) recovery after mitochondrial stressor removal (Fig. 3a), suggesting the involvement of both InsP3R and ryadine receptor channels in ER \(\rightarrow\) lysosome \([\text{Ca}^{2+}]_i\) refling in mitophagy. We next studied the effect of ER \([\text{Ca}^{2+}]_i\) exit channel blockade on \([\text{Ca}^{2+}]_i\), increase by mitochondrial stressors. Both Xesto-spongin C and Dantrolene significantly attenuated \([\text{Ca}^{2+}]_i\), increases by mitochondrial stressors (Fig. 3b), suggesting that ER \(\rightarrow\) lysosome \([\text{Ca}^{2+}]_i\) refling contributes to the increase of \([\text{Ca}^{2+}]_i\), by mitochondrial stressors. When we chelated ER \([\text{Ca}^{2+}]_i\) with a membrane-permeant chelator N,N,N’-tetrakis (2-pyridylmethyl)ethylene diamine (TPEN) that cannot chelate cytosolic \([\text{Ca}^{2+}]_i\) due to low \([\text{Ca}^{2+}]_i\) affinity\(^{21}\), both \([\text{Ca}^{2+}]_i\) recovery after mitochondrial stressor removal and \([\text{Ca}^{2+}]_i\), increase by mitochondrial stressors were reduced (Fig. 3a, b), supporting the role of ER \([\text{Ca}^{2+}]_i\) in these processes. Furthermore, the number of Mito-Keima puncta after rotenone or O/A treatment was significantly reduced by Xesto-spongin C or Dantrolene (Fig. 3c), probably due to insufficient \([\text{Ca}^{2+}]_i\) refling. However, these results do not confirm the role of ER \([\text{Ca}^{2+}]_i\) in these processes because Xesto-spongin C or Dantrolene might have off-target effects and TPEN can chelate \(Zn^{2+}\) in addition to ER \([\text{Ca}^{2+}]_i\). To further corroborate the role of ER \([\text{Ca}^{2+}]_i\) in \([\text{Ca}^{2+}]_i\), recovery after mitochondrial stressor removal, we treated cells with Thapsigargin emptying ER \([\text{Ca}^{2+}]_i\) store\(^{20}\). After Thapsigargin pretreatment, \([\text{Ca}^{2+}]_i\), recovery after mitochondrial stressor removal was abrogated (Supplementary Fig. 6a), supporting the role of ER \([\text{Ca}^{2+}]_i\) in \([\text{Ca}^{2+}]_i\), recovery and ER \(\rightarrow\) lysosome \([\text{Ca}^{2+}]_i\) refling.

Since these results suggest ER \(\rightarrow\) lysosome \([\text{Ca}^{2+}]_i\) movement during mitochondrial stress, we next measured ER \([\text{Ca}^{2+}]_i\) content \([\text{Ca}^{2+}]_i\) during mitophagy in INS-1 cells transfected with \(\text{GEM-CEPIA}1\)\(^{23}\). When \([\text{Ca}^{2+}]_i\) was determined after rotenone or O/A treatment in a Ca\(^{2+}\)-free Krebs-Ringer bicarbonate (KRB) buffer inhibiting possible store-operated Ca\(^{2+}\) entry (SOCE), a significant decrease of \([\text{Ca}^{2+}]_i\) was observed (Supplementary Fig. 6b), consistent with ER \(\rightarrow\) lysosome \([\text{Ca}^{2+}]_i\) refling. \([\text{Ca}^{2+}]_i\) determined using a FRET-based \(\text{DIER}\) probe allowing ratiometric measurement\(^{24}\) also demonstrated a significantly reduced \([\text{Ca}^{2+}]_i\) after rotenone or O/A treatment in a Ca\(^{2+}\)-free KRB buffer (Fig. 3d). Importantly, \([\text{Ca}^{2+}]_i\) was not changed by rotenone or O/A treatment in the presence of extracellular Ca\(^{2+}\) (Supplementary Fig. 6c). These results...
eliminate possible role of sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) inhibition due to rotenone- or O/A-induced decrease of ATP in [Ca\(^{2+}\)]\(_{\text{ER}}\) decrease after mitochondrial stressor treatment without extracellular Ca\(^{2+}\) (see Fig. 3d), since [Ca\(^{2+}\)]\(_{\text{ER}}\) would be decreased by SERCA inhibition even with extracellular Ca\(^{2+}\).

To study dynamic changes in [Ca\(^{2+}\)]\(_{\text{ER}}\) and their temporal relationship with lysosomal Ca\(^{2+}\) refilling, we simultaneously traced [Ca\(^{2+}\)]\(_{\text{ER}}\) and [Ca\(^{2+}\)]\(_{\text{Lys}}\) in cells transfected with GEM-CEPIA1er and loaded with Oregon Green 488 BAPTA-1 Dextran. When cells were treated with rotenone or O/A and organelle [Ca\(^{2+}\)] of cells with reduced [Ca\(^{2+}\)]\(_{\text{Lys}}\) was monitored without rotenone or O/A in a Ca\(^{2+}\)-free KRB buffer, [Ca\(^{2+}\)]\(_{\text{ER}}\) decrease occurred simultaneously with [Ca\(^{2+}\)]\(_{\text{Lys}}\) recovery (Fig. 3e), strongly supporting ER → lysosome Ca\(^{2+}\) refilling by mitochondrial stress. Without mitochondrial stressor removal, [Ca\(^{2+}\)]\(_{\text{Lys}}\) recovery could not be observed probably due to ongoing lysosomal Ca\(^{2+}\) release (Supplementary Fig. 6d).
Fig. 2 Role of Ca\(^{2+}\) and calcineurin in mitophagy. a Tfеб-GFP-transfectants were transfected with pcDNA 3.1-HA or -HA-ΔCnA-H51SIQ. After rotenone or O/A treatment for 4 h, cells were subjected to immunofluorescence with anti-HA Ab. (scale bar, 20 μm) (white arrow, HA-ΔCnA-H51SIQ-transfected cells showing no Tfеб transfection by rotenone or O/A; yellow arrow, untransfected cells showing Tfеб translocation by rotenone or O/A; magenta arrow, control-transfected cells showing Tfеб translocation by rotenone or O/A). b The percentage of cells showing nuclear Tfеб in cells of (a). (scale bar, 20 μm) (n = 8 for HA + Rot or HA + O/A; n = 9 for HA + Veh; n = 19 for HA-ΔCnA-H51SIQ + Rot; n = 20 for HA-ΔCnA-H51SIQ + O/A; n = 21 for HA-ΔCnA-H51SIQ + Veh) e mRFP-LC3-transfectants were transfected with pcDNA 3.1-HA or -HA-ΔCnA-H51SIQ. After rotenone or O/A treatment for 24 h, cells were subjected to immunofluorescence with anti-TOM20 and -HA Abs. (scale bar, 10 μm) d The number mRFP-LC3 puncta colocalized with TOM20 in cells of (c). (n = 7 for HA + Veh, HA + Rot, HA-ΔCnA-H51SIQ + Rot or HA-ΔCnA-H51SIQ + O/A; n = 8 for HA-ΔCnA-H51SIQ + Veh; n = 9 for HA + O/A). e During GTP treatment of GCaMP3-M3L-transfected cells with or without rotenone or O/A pretreatment, perixosomal fluorescence was traced (right upper). Peak fluorescence (right lower). Representative fluorescence images (left). (scale bar, 20 μm) (n = 5) f After mitochondrial stressor treatment for 4 h, [Ca\(^{2+}\)]\(_{\text{i}}\) was determined by Oregon Green 488 BAPTA-1 Dextran loading. Fluorescence intensity (right). Representative fluorescence images (left). (scale bar, 20 μm) (n = 7) g During GTP treatment of Fura-2-loaded cells with or without rotenone or O/A pretreatment, 340/380 nm fluorescence ratio was monitored (left). Lysosomal Ca\(^{2+}\) reservoir estimated by calculating AUC of the curve (right). (n = 14) h After treatment with mitochondrial stressors for 1 h, [Ca\(^{2+}\)]\(_{\text{i}}\) was determined using Fura-2. (n = 10) i After mitochondrial stressor treatment for 4 h in the presence or absence of BAPTA-AM, Tfeb-GFP nuclear translocation was determined (right). Representative fluorescence images (left). (scale bar, 20 μm) (n = 5) Cells in the rectangles were magnified. All data in this figure are the means ± SEM from more than 3 independent experiments. P values were determined using one-way ANOVA with Tukey’s test. *, compared to Veh-treated cells; †, compared to INS-1 cells or HA transfectants treated with Rot alone; ‡, compared to INS-1 cells or HA transfectants treated with O/A alone.

Since ER → lysosome Ca\(^{2+}\) refilling can be facilitated by membrane contact between organelles25, we studied possible apposition of ER and lysosome proteins using proximity ligation assay (PLA). Confocal microscopy after incubation with specific antibodies (Abs) and rolling circle amplification showed close contact between VAPA on ER and ORP11 on lysosome26 by rotenone or O/A (Fig. 3f), suggesting the formation of membrane contact sites between ER and lysosome by mitochondrial stressors, likely facilitating ER → lysosome Ca\(^{2+}\) refilling. We employed another method detecting ER-lysosome contact-a modification of Contact-1D technology using a split–pair system of pBir biotin ligase27. When cells transfected with Flag-BirA(N-G78)-VAPA and STARD3-BirA(G79-C)-HA were treated with rotenone or O/A, biotin conjugate formation was observed after incubation with FITC-streptavidin, indicating contact between ER (VAPA) and lysosome (STARD3) (Supplementary Fig. 6e).

We next studied possible STIM1 oligomerization which can occur after ER Ca\(^{2+}\) emptying and lead to SOCE activation through Ca\(^{2+}\) release-activated channel (CRAC)28. Indeed, STIM1 puncta were clearly observed after rotenone or O/A treatment (Supplementary Fig. 7a), supporting ER Ca\(^{2+}\) emptying by mitochondrial stressors. Furthermore, STIM1 puncta colocalized with Orai1, suggesting Ca\(^{2+}\) influx through SOCE29 during mitophagy (Supplementary Fig. 7a). To confirm the role of extracellular Ca\(^{2+}\) influx through Orai1 in mitophagy, we employed CRAC inhibitors. When we treated cells with BTP2 inhibiting Orai30,31, mitophagy by rotenone or O/A was significantly reduced (Supplementary Fig. 7b). When we depleted extracellular Ca\(^{2+}\) content using 2 mM EGTA reducing [Ca\(^{2+}\)] can be approximated to 161 nM (https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/machecedor/CalGTA-TS.htm)32, mitophagy by rotenone or O/A was again significantly reduced (Supplementary Fig. 7b), supporting the importance of extracellular Ca\(^{2+}\) influx in mitophagy. [Ca\(^{2+}\)]\(_{\text{i}}\) recovery after mitochondrial stressor removal was also reduced by incubation in a Ca\(^{2+}\)-free KRB buffer or 2 mM EGTA (Supplementary Fig. 6a), suggesting the role of extracellular Ca\(^{2+}\) influx in ER → lysosome Ca\(^{2+}\) refilling.

Role of Tfеб and mitophagy in β-cell function. We next studied in vivo role of TFEB-mediated mitophagy in pancreatic β-cell function. To this end, we generated Tfеб\(^{-}\)β-cell mice by crossing Tfеб\(^{-}\)Flox mice to RIP-Cre mice, and imposed metabolic stress by feeding HFD. In this model, we wanted to confirm that metabolic stress can cause mitochondrial stress leading to mitophagy. We thus treated INS-1 cells with palmitic acid (PA), the most abundant saturated fatty acid in vivo and an effector of insulin resistance or β-cell failure33,34. PA treatment of INS-1 cells induced mitochondrial stress, as shown by mitochondrial ROS accumulation (Fig. 4a). PA treatment of INS-1 cells also induced a decrease of [Ca\(^{2+}\)]\(_{\text{i}}\)35 and an increase of [Ca\(^{2+}\)]\(_{\text{m}}\)36,37, supporting that PA-induced mitochondrial ROS influences lysosomal Ca\(^{2+}\) channel. Lysosomal pH was not changed by PA treatment of INS-1 cells (Supplementary Fig. 2b), suggesting that PA-induced mitochondrial ROS influences but decreased by PA (Supplementary Fig. 2d), consistent with previous results35,36 and suggests that changes of intracellular milieu and associated cellular responses after mitochondrial stressor treatment might affect flux of autophagy or selective autophagy differently. ER-,phagic flux represented as red puncta after mCherry-GFP-RAMP4 transfection38 was also not increased but decreased by PA (Supplementary Fig. 2d), consistent with a previous paper38.

While these results suggested the role of PA-induced mitochondrial ROS in mitophagy, PA can affect other signaling pathway such as fatty acid receptors belonging to G protein-coupled receptors (GPCRs) family39 or can be converted to other lipid intermediates through palmitoyl-CoA or sphingosine/ceramide pathway40. We thus examined the effect of inhibitors of such pathways. Pretreatment with DC260126 (a GPCR antagonist)39, Triacsin C (an acyl-CoA synthetase inhibitor), Fumonisin B1 (a sphingosine N-acetyltransferase inhibitor) or Myriocin (an inhibitor of serine palmitoyltransferase, thus ceramide synthesis)40 did not reduce PA-induced mitochondrial ROS accumulation or TFEB nuclear translocation (Supplementary Fig. 8a, b), suggesting that mitochondrial ROS accumulation by PA itself is the most likely event leading to mitophagy.

Since effector molecules of metabolic stress such as PA can induce mitophagy through TFEB activation in vitro, we investigated whether mitophagy occurs in vivo after HFD feeding in pancreatic islet cells expressing Parkin (Supplementary Fig. 1c). Indeed, colocalization of LC3, an autophagic marker, with...
TOM20 in pancreatic islets of Tfeb<sup>F/F</sup> mice assessed by Pearson’s correlation analysis was markedly increased by HFD (Fig. 5a), indicating mitophagy. Intriguingly, increased mitophagy in islets of HFD-fed mice was significantly reduced by β-cell Tfeb KO (Fig. 5a), suggesting TFEB-dependent mitophagy by metabolic stress. As the number of LC3 autophagosomes might not reflect autophagic or mitophagic activity, we studied TOM20 colocalization with a lysosomal marker, as an indicator of mitophagic activity<sup>41</sup>. Mitophagic activity estimated by colocalization of TOM20 with LAMP2 was markedly increased in islets of HFD-fed Tfeb<sup>F/F</sup> mice. Furthermore, the increased mitophagic activity in islets of HFD-fed mice was significantly reduced by β-cell Tfeb KO (Fig. 5b), suggesting TFEB-dependent increase in β-cell mitophagic activity by HFD. Electron microscopy (EM)
also demonstrated decreased aspect ratio of mitochondria in β-cells of HFD-fed TfebF/F mice, suggestive of mitophagy, which was reversed by β-cell Tax1bp1 KO (Supplementary Fig. 9a). The number of mitochondria surrounded by isolation membrane or autophagosome in β-cells of HFD-fed TfebF/F mice was also significantly increased compared to that of normal chow diet (NCD)-fed TfebF/F or HFD-fed TfebΔβ-cell mice (Supplementary Fig. 9b). In contrast, autophagic activity determined by colocalization between LAMP2 and LC3 was not significantly changed by HFD feeding or Tfeb KO (Supplementary Fig. 9c), which is in agreement with no increase of autophagic activity by PA in vitro and increased mitochondrial activity without increased autophagic activity in cardiac tissue by HFD for 2 months. The number of autophagosome counted by EM was also not changed by HFD or Tfeb KO (Supplementary Fig. 9d).

Probably due to decreased mitochondria, ROS accumulation detected by 4-hydroxynonenal (HNE) staining that was increased in islets of TfebF/F mice on HFD was further increased by β-cell Tfeb KO (Fig. 5c). Regarding mitochondrial activity, cytochrome c oxidase (COX) activity in pancreatic islets was increased by HFD probably as an adaptation to metabolic stress, which was significantly reduced by β-cell Tfeb KO (Fig. 5d), suggesting Tfeb-dependent functional mitochondrial adaptation to metabolic stress. Seahorse respirometry also showed significant increases of basal, glucose-stimulated, ATP-coupled and maximal O2 consumption in pancreatic islets of HFD-fed mice again probably as an adaptation to metabolic stress. These increases of O2 consumption were abolished by Tfeb KO (Fig. 5e), supporting Tfeb-dependent adaptive response to metabolic stress.

Metabolically, NCD-fed TfebΔβ-cell mice showed no abnormality in glucose profile up to 20 weeks of age (Supplementary Fig. 10a). However, HFD-fed TfebΔβ-cell mice showed aggravated glucose intolerance compared to HFD-fed TfebF/F mice, judged by increased AUC of glucose tolerance test (GTT) (Fig. 6a), while non-fasting blood glucose during or after HFD feeding for 20 weeks did not significantly differ between them (Supplementary Fig. 10a). Aggravated glucose intolerance of HFD-fed TfebΔβ-cell mice was accompanied by reduced β-cell function compared to HFD-fed TfebF/F mice as shown by decreased insulinogenic index (Fig. 6b) without significant changes of β-cell mass (Supplementary Fig. 10b), suggesting that Tfeb-KO β-cells could not functionally adapt to increased demand for insulin due to insufficient mitophagy and mitochondrial activity, leading to deteriorated glucose intolerance when metabolic stress was imposed. In contrast, AUC of insulin tolerance test (ITT) and body weight did not differ between TfebΔβ-cell and TfebF/F mice on either NCD or HFD (Supplementary Fig. 10c, d).

While these results demonstrated the role of Tfeb in β-cell function and metabolic profile, Tfeb3 is that is activated by mitochondrial stress might also play a non-redundant role in β-cell response to metabolic stress. We thus crossed TfebΔβ-cell mice to Tfeb3-KO mice. While TfebF/F/Tfeb3-KO and TfebΔβ-cell/Tfeb3-KO mice had slightly impaired glucose tolerance compared to TfebF/F/Tfeb3-WT mice and TfebΔβ-cell/Tfeb3-WT, respectively, these differences were statistically insignificant in both NCD- and HFD-fed conditions (Supplementary Fig. 10e), suggesting no further independent role of endogenous TFEB3 in metabolic adaptation of β-cells.

Using TfebΔβ-cell mice, we studied which genes are differentially regulated by Tfeb. Expression of Tfeb downstream genes related to autophagy such as LC3 (Map1lc3b), Clcn7 and Ctsa was upregulated in pancreatic islets of HFD-fed mice together with increased expression of Tfeb and Tfeb3 themselves (Fig. 6c). Increased expression of these genes was significantly reduced by β-cell Tfeb KO (Fig. 6d), suggesting Tfeb-dependent downstream gene induction in pancreatic islets by HFD. Intriguingly, expression of Ndp52 and Optn, crucial autophagy receptors related to mitophagy, was significantly increased in pancreatic islets of HFD-fed TfebF/F mice, and these increases were markedly reduced by β-cell Tfeb KO (Fig. 6c), which could be related to Tfeb-dependent increase of mitophagy by HFD. Expression of other potential mitophagy receptors, such as Nbr1, Tbk1 or Taxilbp1, in pancreatic islets was also increased by HFD, but these increases were statistically insignificant (Fig. 6c). Intriguingly, the expression of mitochondrial biogenesis genes such as Tfam, ESRRα or PGC-1α was increased in pancreatic islets of TfebF/F mice by HFD. Such induction of mitochondrial biogenesis genes by HFD was abrogated by β-cell Tfeb KO (Fig. 6c), which is similar to previous results showing Tfeb-dependent induction of mitochondrial biogenesis genes in other tissues. Probably due to increased mitochondrial biogenesis in pancreatic islet cells by HFD, normalized density of TOM20 band in islets of TfebF/F mice on HFD was not different from that of the same mice on NCD despite significantly increased mitophagy which can reduce mitochondrial proteins including TOM20 (Supplementary Fig. 10f).

We next imposed mitochondrial stress on pancreatic islets ex vivo. In rotenone- or O/A-treated islets, mRNA expression of Ndp52, Optn and other autophagy or lysosomal genes such as Ctsa, Atg6v1h, Tfeb or Tfeb3 was significantly increased (Fig. 6d). Furthermore, increases in Ndp52, Optn, Tfeb and Atg6v1h...
Fig. 4 Effect of PA on mitochondria and mitophagy. 

a After treating INS-1 cells with PA for 2-24 h, mitochondrial ROS was determined using MitoSOX (right). Representative flow cytometric scattergrams are presented (left). (n = 4) b After treating cells with PA for 1 h, [Ca^{2+}]_{i} was determined by Oregon Green 488 BAPTA-1 Dextran loading (right). Representative fluorescence images are presented (left). (scale bar, 20 μm) (n = 9) c After treating cells with PA for 1 h, [Ca^{2+}]_{i} was determined using Fura-2 (n = 10) d After treating Tfeb-GFP-transfected cells with PA for 4-16 h, nuclear TFEB was examined by confocal microscopy (right). Representative fluorescence images are presented (left). (scale bar, 20 μm) (n = 10 for 16 h; n = 11 for Veh; n = 12 for 4 h or 6 h) e After treating pMito-Keima-transfected cells with PA for 18 h, the number of red acidic puncta in transfected cells was determined (right). Representative fluorescence images are presented (left). (scale bar, 5 μm) (n = 7) f After PA treatment for 4 h with or without ROS quencher pretreatment for 1 h, nuclear TFEB was examined as in (d) (lower). Representative fluorescence images are presented (upper). (scale bar, 20 μm) (n = 6 for MitoTEMPO; n = 7 for NAC; n = 11 for Veh, PA, NAC + PA or MitoTEMPO + PA) Cells in the rectangles were magnified. All data in this figure are the means ± SEM from more than 3 independent experiments. P values were determined using one-way ANOVA with Tukey’s test (a, d, f) or two-tailed t test (b, c, e). *, compared to Veh-treated cells; †, compared to cells treated with PA alone.
expression by ex vivo rotenone or O/A treatment were significantly reduced by β-cell Tfeb KO (Fig. 6d), supporting that TFEB-dependent induction of crucial mitophagy receptors and lysosomal genes by mitochondrial or metabolic stress facilitates mitophagy.

Since these results suggest that Ndp52 and Optn are potential TFEB targets, we conducted chromatin immunoprecipitation (ChIP) assay. To this end, we studied promoters of human NDP52 and OPTN that are well characterized. When 1.1B4 human islet cells generated by electrofusion of human primary pancreatic islet cells to pancreatic adenocarcinoma cells were treated with rotenone or O/A, NDP52 or OPTN expression was significantly increased, which was downregulated by siTFEB (Fig. 6e, f). Contrarily, TFEB-GFP transfection significantly increased NDP52 or OPTN expression in 1.1B4 cells (Fig. 6g), supporting the validity of 1.1B4 cells to this end. In rotenone- or O/A-treated 1.1B4 cells, TFEB binding to putative Coordinated Lysosomal Expression and Regulation (CLEAR) sites of NDP52 or OPTN promoter was significantly increased (Fig. 6h), supporting that NDP52 and OPTN are TFEB targets. Since Nfe2l2 encoding nuclear factor erythroid 2–related factor 2 (Nrf2) can be activated by TFEB or ROS, and Ndp52 and Optin could be...
Fig. 5 Effect of in vivo Tfeb KO on mitophagy in β-cells. a Colocalization of LC3 puncta with TOM20, a mitochondrial marker, in pancreatic islets of TfebΔβ-cell and TfebΔβ/Δβ mice fed NCD or HFD for 12 weeks estimated by Pearson’s correlation analysis (right). Representative fluorescence images are presented (left). (scale bar, 10 μm) (n = 6) b Colocalization of LAMP2 spot, a lysosomal marker, with TOM20 in pancreatic islets of TfebΔβ-cells and TfebΔβ/Δβ mice fed NCD or HFD for 12 weeks estimated by Pearson’s correlation analysis (right). Representative fluorescence images are presented (left). (scale bar, 10 μm) (n = 6) c ROS accumulation determined by HNE staining of pancreatic sections from TfebΔβ-cell and TfebΔβ/Δβ mice fed NCD or HFD for 12 weeks (right). Representative fluorescence images are presented (left). (scale bar, 50 μm) (n = 7 for TfebΔβ-cells+HFD; n = 8 for TfebΔβ-cells+NCD; n = 11 for TfebΔβ/Δβ-NCD; n = 14 for TfebΔβ/Δβ-HFD) d Mitochondrial COX activity in pancreatic islets of TfebΔβ-cells and TfebΔβ/Δβ mice fed NCD or HFD for 12 weeks (right). Representative DAB images are presented (left). (scale bar, 100 μm) (n = 6) e Seahorse XF respirometry using primary pancreatic islets from TfebΔβ-cell and TfebΔβ/Δβ mice on NCD or HFD for 12 weeks (left). Basal, glucose-stimulated, ATP-coupled and maximal O₂ consumptions were calculated from the curves (right). (OCR, O₂ consumption rate; FCCP, carbonyl cyanide-4-[trifluoromethoxy]phenylhydrazone; Rot, rotenone; AA, antimycin A) Cells in the rectangles were magnified.

Previous studies have shown the role of Nfe2l2 target genes in mitigating mitochondrial stressors. We studied the role of Nfe2l2 in Ndp52 or Optn expression employing pancreatic islets from Nfe2l2-KO mice. Induction of Ndp52 or Optn in primary islet cells by rotenone or O/A was not significantly reduced by Nfe2l2 KO (Fig. 6i), suggesting that mitochondrial stressor-induced Ndp52 and Optn in pancreatic islet cells is largely due to TFEB-mediated transactivation. These results indicate that TFEB-dependent mitophagy, contributed by transactivation of mitophagy receptor genes such as Ndp52 or Optn, plays an important role in β-cell adaptation to metabolic stress through improved mitochondrial function.

Since the induction of Ndp52 and Optn by metabolic or mitochondrial stressors could explain selective mitophagy observed in pancreatic islets of HFD-fed mice, we conducted KD of Ndp52 and Optn in INS-1 cells that show increased expression of Ndp52 or Optn after treatment with PA (Supplementary Fig. 6a). Indeed, KD of Ndp52 and Optn that successfully reduced expression of Ndp52 and Optn, respectively (Supplementary Fig. 6b), significantly reduced mitophagy after PA treatment (Supplementary Fig. 6c), suggesting that induction of Ndp52 or Optn in β-cells contributes to the selective mitophagy after HFD feeding. The role of Ndp52 and Optn in mitophagy after rotenone or O/A treatment was also studied using INS-1 cells that show elevated expression of Ndp52 and Optn after rotenone or O/A treatment (Supplementary Fig. 6d). Mitophagy after rotenone or O/A treatment was also significantly reduced by KD of Ndp52 or Optn. Combined KD of both Ndp52 and Optn further reduced mitophagy after rotenone or O/A treatment (Supplementary Fig. 6e), suggesting that induction of Ndp52 or Optn contributes to mitophagy after treatment with mitochondrial stressors.

Discussion

Previous studies have shown the role of Tfeb family in systemic metabolism and mitochondrial function and systemic metabolism has not been studied. Furthermore, while the role of autophagy in β-cell function and metabolism has been studied extensively, the role of mitophagy in β-cell function has been hardly investigated.

Mitophagy of pancreatic β-cells appears to be important in mitochondrial integrity and functional adaptation during metabolic or mitochondrial stress because HFD feeding increased insulinogenic index in wild-type mice but not in TfebΔβ-cell mice that showed defective mitophagic response. Although the role of other types of selective autophagy or bulk autophagy modulated by Tfeb cannot be disregarded in Tfeb-mediated protection of β-cell function, flux of autophagy or ER-phagy was not increased by PA or HFD feeding. These results are consistent with previous papers showing no increase in fluxes of autophagy or ER-phagy in vitro and in vivo by PA or HFD feeding, which could be due to different intracellular environment and associated cellular responses imposed by diverse organelle stressors such as induction of mitophagy receptors, modifying TFEB-dependent or -independent autophagic activity.

Tfeb-mediated mitophagy appears to be important in adaptive mitochondrial respiration because HFD-induced upregulation of mitophagy, COX activity, and mitochondrial O₂ consumption in β-cells was abolished by Tfeb KO. Increases in mitophagy, COX activity, and mitochondrial O₂ consumption in pancreatic islets of HFD-fed mice are in line with previous results reporting increased mitochondrial respiration, complex activities, or content and mitophagy in the heart or islet tissues after HFD feeding, which could be due to fatty acid-induced uncoupling, O₂ shunting to ROS or adaptive changes to metabolic stress. Probably due to impaired mitophagy and mitochondrial adaptation to metabolic stress, HFD-induced ROS accumulation was augmented by β-cell Tfeb KO, leading to significantly suppressed β-cell function and aggravated glucose intolerance. Consistent with HFD-induced increases of mitophagy and COX activity, induction of Tfeb downstream genes or Tfeb and Tfe3 themselves was observed in pancreatic islets of HFD-fed mice, again supporting the role of Tfeb activation in adaptation to HFD. However, we observed no further metabolic deterioration by additional Tfe3 KO despite comparable Tfe3 activation by mitochondrial stressors, suggesting no non-redundant role of Tfe3 in β-cell adaptation to metabolic stress. These results are in contrast to a previous paper showing an independent role of Tfe3 in metabolic adaptation.

Such discrepancies could be due to different types of tissues analyzed and non-identical experimental methods or mouse background.

Although these results suggest important roles of mitophagy in pancreatic β-cell function, previous papers have reported no significant decrease of glucose-stimulated insulin release from β-cells with targeted disruption of Parkin or PINK1, an upstream kinase recruiting Parkin onto dysfunctional or damaged mitochondria. This discrepancy could be attributable to compensatory changes in Parkin- or PINK1-KO β-cells which might include Parkin-independent mitophagy such as piecemeal-type mitophagy or receptor-mediated mitophagy that can occur in Parkin-KO or PINK1-KO β-cells. In contrast, Parkin-KD INS-1 cells showed diminished mitochondrial function and glucose-stimulated insulin release, which might be due to the absence of compensatory changes associated with Parkin KO. The role of β-cell Parkin in the maintenance of mitochondrial function and glucose-stimulated insulin release after treatment with streptozotocin, a classical β-cell toxin, has also been reported, suggesting the role of Parkin-mediated mitophagy of β-cells in stressed condition.

We observed Ndp52 and Optn induction in pancreatic islets by metabolic or mitochondrial stress, which was attenuated by Tfeb.
KO. Since NDP52 and OPTN are primary receptors for mitophagy\(^{46}\), TFEB-dependent induction of Ndp52 and Optn in pancreatic islet cells is likely to contribute to the increased mitophagy by metabolic stress or mitochondrial stress. These results suggest a role of transactivation of Ndp52 and Optn in mitochondrial stress-induced mitophagy besides mitophagy receptor phosphorylation\(^{63}\).

When we studied the mechanism of mitophagy in β-cells undergoing mitochondrial stress, we observed significantly increased \([\text{Ca}^{2+}]_i\), by rotenone or O/A, similar to results using other cell types\(^{64,65}\). As antimycin A had a relatively weak effect on mitochondrial potential, it has been used in combination with oligomycin inhibiting reverse activity of F\(_{1}\)F\(_{0}\)-ATPase\(^{66}\). Ca\(^{2+}\)-channel responsible for lysosomal Ca\(^{2+}\) release by mitochondrial...
ROS appears to be TRPML1. Besides PI(3,5)P2, ROS can also activate TRPML1 channel but not TRPML2 or TRPML3 channel. Since ML-S13 is not specific for TRPML1, ML-S13-mediated abrogation of mitochondrial stressor-induced \( [\text{Ca}^{2+}]_\text{Lys} \), increase does not prove the role of TRPML1 in TFEB activation and mitophagy by mitochondrial stressors. However, lysosomal \( [\text{Ca}^{2+}]_\text{Lys} \) release by mitochondrial ROS that does not activate TRPML2 or TRPML3 channel and abrogation of mitochondrial stressor-induced TFEB activation by \( \text{sir} \text{Mcoln1} \) strongly support the role of TRPML1 in mitochondrial stressor-induced lysosomal \( [\text{Ca}^{2+}]_\text{Lys} \) release. Following lysosomal \( [\text{Ca}^{2+}]_\text{Lys} \) release through TRPML1, increased \( [\text{Ca}^{2+}]_\text{Lys} \), dephosphorylated TFEB and TFE3 via calcineurin activation. We also observed notably decreased total TFEB and TFE3 following O/A treatment. Although the mechanism of this phenomenon is unknown, it could be due to changes in the phosphorylation of amino acid residues related to the stability of TFEB family members.

Although the initial lysosomal \( [\text{Ca}^{2+}]_\text{Lys} \) release occurs in response to mitochondrial ROS, small lysosomal \( [\text{Ca}^{2+}]_\text{Lys} \) reservoir might not be sufficient to accomplish full progression of mitophagy. While \( [\text{Ca}^{2+}]_\text{Lys} \) is comparable to \( [\text{Ca}^{2+}]_\text{ER} \), lysosome is not a major \( [\text{Ca}^{2+}]_\text{Lys} \) reservoir because its volume is 1/10 of \( [\text{Ca}^{2+}]_\text{ER} \). Previous papers demonstrated occurrence of ER \( \rightarrow \) lysosome \( [\text{Ca}^{2+}]_\text{ER} \) refilling for completion of events requiring lysosomal \( [\text{Ca}^{2+}]_\text{Lys} \). ER \( [\text{Ca}^{2+}]_\text{ER} \) channels such as \( \text{InsP}3R \) or ryanodine receptor channel could act as ER \( [\text{Ca}^{2+}]_\text{ER} \) exit channels in ER \( \rightarrow \) lysosome \( [\text{Ca}^{2+}]_\text{ER} \) refilling. Indeed, genetic studies have shown the participation of \( \text{InsP}3R \) in mitophagy; however, the role of ER \( [\text{Ca}^{2+}]_\text{ER} \) or \( \text{InsP}3R \) in lysosomal \( [\text{Ca}^{2+}]_\text{ER} \) refilling and the relationship between ER \( [\text{Ca}^{2+}]_\text{ER} \) and lysosomal \( [\text{Ca}^{2+}]_\text{Lys} \) in mitophagy have not been studied. Our data show that ER \( \rightarrow \) lysosome \( [\text{Ca}^{2+}]_\text{ER} \) refilling occurs during mitochondrial stress, which is necessary for replenishment of lysosomal \( [\text{Ca}^{2+}]_\text{Lys} \) reservoir and completion of TFEB activation or mitophagy.

ER \( \rightarrow \) lysosome \( [\text{Ca}^{2+}]_\text{Lys} \) refilling could be facilitated by ER-lysosome contact. In addition to well-characterized ER-mitochondrial contact, ER-lysosome contact could have a functional role in cellular responses. When ER \( [\text{Ca}^{2+}]_\text{ER} \) emptying due to ER \( \rightarrow \) lysosome flux, SOCE is likely to occur through CRAC activation, which is characterized by STIM1 oligomerization and translocation to ER-plasma junction where it interacts with Orai channels. Thus, STIM1 oligomerization by mitochondrial stressors strongly supports that ER \( [\text{Ca}^{2+}]_\text{ER} \) emptying occurs due to ER \( \rightarrow \) lysosome \( [\text{Ca}^{2+}]_\text{ER} \) flux, which also can explain the suppression of mitophagy by extracellular \( [\text{Ca}^{2+}]_\text{ER} \) depletion.

Altogether, these results suggest that TFEB is activated by lysosomal \( [\text{Ca}^{2+}]_\text{Lys} \) release coupled with ER \( \rightarrow \) lysosome \( [\text{Ca}^{2+}]_\text{ER} \) refilling during mitochondrial or metabolic stress, and TFEB-dependent induction of mitophagy, facilitated by transactivation of mitophagy receptors such as \( \text{Ndp}52 \) or \( \text{Optn} \), plays an important role in \( \beta \)-cell adaptation to metabolic stress through improved mitochondrial function.

**Methods**

**Detection of mitophagy in vitro.** To visualize mitophagy using a mitophagy-specific probe, INS-1 cells were transfected with a pMito-Keima (MBL), and then treated with rotenone or O/A for 18 h. Keima fluorescence at neutral pH was observed at an excitation wavelength of 430 ± 20 nm and an emission wavelength of 424 ± 20 nm. Keima fluorescence in acidic conditions was observed at an excitation wavelength of 562 ± 20 nm and emission wavelength of 540 ± 20 nm. To visualize mitophagy in another way, cells transfected with the mRFP-LC3 construct were treated with mitochondrial stressors and then incubated with anti-TOM20 Ab (Cell Signaling Technology, 1:200). Images were obtained using an LSM780 confocal microscope (Zeiss) from more than five different fields for each experiment. Colocalization of puncta or fluorescence in vitro was evaluated manually in a blinded manner.

**GCaMP3 Ca\(^{2+}\) imaging.** INS-1 cells were transfected with a plasmid encoding a perisomal GCaMP3-M1 Ca\(^{2+}\) probe. After 48 h, lysosomal Ca\(^{2+}\) release was measured in a zero Ca\(^{2+}\) solution (145 mM NaCl, 5 mM KCl, 3 mM MgCl\(_2\), 10 mM glucose, 1 mM EGTA and 20 mM HEPES, pH 7.4) with or without rotenone or O/A pretreatment for 1 h by measuring fluorescence intensity at 470 nm with a LSM780 confocal microscope (Zeiss). GPN was added at the indicated time.

**Cytosolic Ca\(^{2+}\) measurement.** For ratiometric measurement of \( [\text{Ca}^{2+}]_\text{cyt} \), INS-1 cells were transfected with 2 \( \mu \)M of the acetoxymethyl ester form of Fura-2 (Invitrogen) in RPMI-1640 at 37 °C for 30 min and then rinsed with fresh solution. Measurements were performed using MetaFluor (Molecular devices) on an Axio Observer A1 (Zeiss) equipped with a 150 W xenon lamp Polychrome V (Till Photonics), a CoolSNAP-Hq2 digital camera (Photometrics), and a Fura-2 filter set. \( [\text{Ca}^{2+}]_\text{cyt} \), was determined in a phenol-free RPMI-1640 using the 340/380 nm fluorescence ratio and the following equation:

\[
[\text{Ca}^{2+}]_\text{cyt} = K_\text{d} x (R - R_{\text{min}})/(R_{\text{max}} - R) 
\]

where \( K_\text{d} \) is Fura-2 dissociation constant (224 mM at 37 °C, \( R_{\text{max}} \) = 380 nm fluorescence in the absence of Ca\(^{2+}\), \( R_{\text{min}} \) = 380 nm fluorescence with saturating Ca\(^{2+}\), \( R \) = 340/380 nm fluorescence ratio, \( R_{\text{max}} \) = 380/380 nm ratio with saturating Ca\(^{2+}\), and \( R_{\text{min}} \) = 340/380 nm ratio in the absence of Ca\(^{2+}\). To monitor \( [\text{Ca}^{2+}]_\text{cyt} \), after GPN treatment as an estimate of lysosomal Ca\(^{2+}\) content, INS-1 cells were incubated in a zero Ca\(^{2+}\) solution containing 145 mM NaCl, 5 mM KCl, 3 mM MgCl\(_2\), 10 mM glucose, 1 mM EGTA and 20 mM HEPES (pH 7.4).

**Measurement of lysosomal Ca\(^{2+}\) content \( ([\text{Ca}^{2+}]_\text{Lys}] \).** To measure \( [\text{Ca}^{2+}]_\text{Lys} \), cells were loaded with 100 \( \mu \)g/mL of Oregon Green 488 BAPTA-1 Dextran (Invitrogen) at 37 °C for 12 h. Medium was then changed to one without Oregon Green 488 BAPTA-1 Dextran, and cells were chased for an additional 4 h. After treating cells with rotenone or O/A for the last 1 h of chasing, cells were washed in HEPES-buffered saline (135 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl\(_2\), 1.5 mM CaCl\(_2\), 11.5 mM glucose, 11.6 mM HEPES, pH 7.3) for confocal microscopy.

**Measurement of ER Ca\(^{2+}\) content \( ([\text{Ca}^{2+}]_\text{ER}] \).** INS-1 cells were transfected with GEM-CPEIA1er (Addgene) or a ratiometric FRET-based Cameleon probe DIVER, using Lipofectamine 2000. After 48 h, cells were treated with rotenone or O/A in a Ca\(^{2+}\)-free KRB buffer (Sigma) for 1 h to inhibit influx of extracellular Ca\(^{2+}\) into ER or in a culture medium for 1 h. GEM-CPEIA1er fluorescence was...
measured using an LSM780 confocal microscope (Zeiss) at an excitation wave-length of 405 nm and emission wavelengths of 466 or 520 nm, and F466/F520 was calculated as ratio of [Ca²⁺]_cyt, DHE, fluorescence intensity ratio (F480/F490) determined using an LSM980 confocal microscope (Zeiss).

Simultaneous monitoring of [Ca²⁺]_cyt and [Ca²⁺]_ER. Twenty-four h after transfection with GEM-CEPA1er, INS-1 cells were loaded with Oregon Green 488 BAPTA-1 Dextran for 12 h and chased for 4 h. After treating cells with rotenone or O/A for 2 h, cells were monitored for [Ca²⁺]_cyt and [Ca²⁺]_ER in a Ca²⁺-free KRB buffer in the presence or absence of rotenone or O/A using an LSM780 confocal microscope.

Lysosomal pH. After incubating with mitochondrial NaoH or PA and 1 μM LysoSensor green DND-189 in a pre-warmed HBS buffer (135 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 1.5 mM CaCl₂, 11.5 mM glucose, 11.5 mM HEPES, pH 7.3) for 10 min in 37°C incubator, LysoSensor green intensity was acquired using a confocal microscope and converted to pH using a standard curve obtained after treating cells with 10 μM nigericin for 20 min in 105 mM KCl buffer (105 mM KCl, 25 mM Hepes, 1 mM MgCl₂) adjusted to different pH 7.2.

Proximity ligation assay (PLA). Contact between ER and lysosome was studied using a Duolink InSitu Detection Reagents kit (Sigma) according to the manufacturer’s protocol. Briefly, cells were incubated with primary Abs against ORP1L (Abcam, 1:200) and VAPA (Santa Cruz, 1:200) at 4°C overnight. After washing, cells were incubated with PLA plus and minus probes at 37°C for 1 h. After ligation reaction to close the circle and rolling circle amplification (RCA) of the ligation product, fluorescence-labeled oligonucleotides hybridized to the RCA products was observed by fluorescence microscopy.

Contact-ID. Full-length DNA fragments of mouse VAPA and STARD3 were produced from cdNA from fJ74A1 (ATCC, TIB-67) cells by PCR. DNA fragments encoding VAPA and STARD3 were inserted into Age1-XhoI site of Flag-BirA(N-G78) and Hind3-BamH1 site of BirA(G79-C)-HA vectors, respectively, using T4 DNA ligase (Takara). Nucleotide sequences of all constructs were confirmed by automatic DNA sequencing. Twenty-four h after transfection of INS-1 cells with Flag-BirA-VAPA and STARD3-BirA using Lipofectamine 2000, 50 μM biotin was added for labeling overnight. After treatment with rotenone or O/A for 1 h, cells were fixed with 4% paraformaldehyde and then permeabilized with 0.5% Triton X-100 PBS. After 10% goat serum blocking, cells were sequentially incubated with anti-FLAG (Sigma, 1:200) and -HA Abs (Cell Signaling Technology, 1:200) diluted in PBS-1% BSA, secondary Abs diluted in PBS-1% BSA for 30 min, and then with FITC-streptavidin diluted in PBS-1% BSA for 30 min at room temperature. (VAPA-Age1 forward: CGACCGGTCATGGCGTCCGCCTCC GGG GCC ATG GC; VAPA-Xho1 reverse: GCCTCGAGCTACAAGATGAATT TCCTAGAGAAGAA; STARD3-Hind3 forward: CCAAGCTTATGAGCAAGCGAGG GGG GCC ATG GC; STARD3-BamH1 reverse: CGATCCCTTACAGGCGC CGCAGGCGCCTACACACG) were separated on 4% polyacrylamide gel (Life Technologies). Immunoblotting was conducted by sequential incubation with anti-GFP (AbFrontier, 1:1,000) or anti-HA (Santa Cruz, 1:1,000) for 1 h. Bands were visualized using an ECL kit for the detection of chemiluminescence.
Measurement of intracellular ROS. To determine intracellular ROS, cells were treated with rotenone or O/A for 1 h after NAC pretreatment for 1 h. After incubation with 5 μM CM-H2DCFDA (Invitrogen) at 37 °C for 30 min in culture medium without PBS and recovery in complete medium for 10 min, confocal microscopy was performed. To study mitochondria-specific ROS, cells were stained with 5 μM MitoSOX (Invitrogen) at 37 °C for 30 min. Cells were suspended in PBS 1%, and then were subjected to flow cytometry by FACSVerse (BD Biosciences) using FlowJo software (TreeStar). The gating strategies for flow cytometric data analysis are shown in Supplementary Fig. 11.

ChIP assay. ChIP was performed according to the manufacturer’s protocol (SimpleChIP® Enzymatic Chromatin IP Kit, Cell Signaling). Briefly, the chromatin/DNA protein complexes were prepared by cross-linking chromatins of TFEB-GFP-transfected 1.1B4 cells treated with vehicle, rotenone, or O/A for 8 h by addition of 1% formaldehyde and incubation at room temperature for 10 min. Cross-linking extracts were then sonicated to shear DNA and immunoprecipitated using anti-GFP (1:1,000) or IgG (1:50) with constant rotation at 4 °C overnight. After the addition of 30 μl of Protein-G bead slurry to the lysates and incubation with rotation at 4 °C for 2 h, resins were washed with the ChIP buffer supplied with the kit. After elution of chromatin from the Ab/Protein-G beads with ChIP Elution Buffer and reversing cross-linking by adding 5 M NaCl and 2 μl of 20 mg/ml proteinase K, DNA was purified for PCR using primers designed to cover the putative TFEF CLEAR sequence (CAGCTG)50 in the human NPD52 (−5,653 from the 5’ UTR) and OPTN promoters (−6885 from the 5’ UTR) (Supplementary Table 4). PCR consisted of 35-40 cycles of denaturation at 97 °C for 30 s, annealing at 65 °C and 55 °C for 30 s, and extension at 72 °C for 40 s for human NPD52 and OPTN promoter, respectively.

Animals. Tfe3fl/fl mice were generated by breeding Tfe3fl/fl (EuROMM/WW) mice (Mutant Mouse Resource and Research Center) with FLPRe mice (Wellcome Trust Sanger Institute). Tfe3fl/fl mice were generated by crossing Tfe3fl/fl mice with RIP-Cre mice (Jackson Laboratory). Eight-week-old male Tfe3fl/fl mice acquired were maintained in a 12 h light/12 h dark cycle and fed HFD for 12 weeks. During the observation period, mice were monitored for glucose profile and weight. Tfe3-KO mice were from the Jackson Laboratory. All animal experiments were conducted in accordance with the Public Health Service Policy in the Human Care and Use of Laboratory Animals. The mouse breeding was fully 22 ± 2 °C, and the humidity was 50 ± 10%. Mouse experiments were approved by the IACUC of the Department of Laboratory Animal Resources of Yonsei University College of Medicine, an AAALAC-accredited unit.

Isolation of pancreatic islets. Primary murine islets were isolated from fasted mice using the collagenase digestion technique1. Briefly, after injecting 2.5 ml of collagenase P (0.8 mg/ml) into the common bile duct, the pancreas was procured and incubated in a collagenase solution at 37 °C for 13 min 20 s. After cessation of enzymatic digestion with cold HBSS-5% FBS, tissue was passed through a 400 μm sieve and then centrifuged on 1,105, 1,085, 1,069, and 1,037 g/ml Biocoll gradients (Biochrom). Islets were collected from the interface using micropipettes.

Intraperitoneal GTT and insulinogenic index. Intraperitoneal GTT was performed by intraperitoneal injection of 1 g/kg of glucose after overnight fasting. Blood glucose concentrations were determined using a One Touch glucometer (Lifescan). Islets were collected from the interface using micropipettes. Islets were collected from the interface using micropipettes.

Immunohistochemistry. Tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Sections of 4 μm thickness were stained for immunohistochemical analyses using anti-LC3 (MBL, 1:200), -LAMP2 (Abcam, 1:200), -TOM20 (Cell Signaling), -Chemcitrate (Abcam, 1:200). Immunofluorescence was visualized with a standard DAB reaction. Images were acquired with a light microscope and analyzed using Fiji11. To evaluate mitophagy in vivo, colocalization between LC3 and TOM20 or that between LAMP2 and TOM20 was assessed by calculating Pearson’s correlation coefficient. To evaluate autophagic flux in vivo, colocalization between LC3 and LAMP2 was determined in the same way.

Bioenergetic analysis of O2 consumption. O2 consumption of isolated mouse islets was measured using a Seahorse Extracellular Flux (XF96) Analyzer (Agilent Technologies) equipped with a spheroid microplate-compatible thermal tray according to a modification of the manufacturer’s protocol. Briefly, islets were seeded into the wells of a poly-L-lysine-coated XF96 spheroid microplate (25 islets/well). Islet seeding was done by inserting a pipette tip directly into the wells of the spheroid microplate. Islets then were incubated with pre-warmed XF assay medium (Seahorse XF base DMEM supplemented with 3 mM glucose, 1% FBS, 1 mM sodium pyruvate and 2 mM glutamine) at 37 °C for 1 h in a non-CO2 incubator. Basal respiration was measured in XF assay medium containing 3 mM glucose. Islets were then sequentially exposed to 20 mM glucose, 5 μM oligomycin, 1 μM FCCP and 1 μM rotenone/antimycin A at the indicated time points to measure glucose-stimulated, ATP-coupled, maximal and non-mitochondrial O2 consumption, respectively. O2 consumption was analyzed using the WaveTM software (Aglent Technologies).

Statistical analysis. All values are expressed as the means ± SEM of ≥ 3 independent experiments performed in triplicate. For densitometric analysis of immunoblot bands, the mean from 2 independent experiments was calculated. Two-tailed Student’s t test was used to compare values between two groups. One-way ANOVA with Tukey’s test was used to compare values between multiple groups. Two-way repeated-measures ANOVA with Bonferroni’s post-hoc test was used to compare multiple repeated measurements between groups. P values < 0.05 were considered to represent statistically significant differences.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The data generated or analyzed during the current study and materials are available from the corresponding author without imposed restriction on reasonable request. Source data are provided with this paper.

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M.-S.L. conceived the experiments. K.P., H.L., J.K., Y.H., Y.S.L., S.H.B., H. K., S.-W.K., and J.Y.K. conducted experiments. M.-S.L. and K.P. wrote the manuscript.

Competing interests
M.-S.L. is the CEO of LysoTech, Inc. All authors declare no competing interests.

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