GADD34 is a modulator of autophagy during starvation

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Cells respond to starvation by shutting down protein synthesis and by activating catabolic processes, including autophagy, to recycle nutrients. This two-pronged response is mediated by the integrated stress response (ISR) through phosphorylation of elf2\textalpha, which represses protein translation, and by inhibition of mTORC1 signaling, which promotes autophagy also through a stress-responsive transcriptional program. Implementation of such a program, however, requires protein synthesis, thus conflicting with general repression of translation. How is this mismatch resolved? We found that the main regulator of the starvation-induced transcriptional program, TFEB, counteracts protein synthesis inhibition by directly activating expression of GADD34, a component of the protein phosphatase 1 complex that dephosphorylates elf2\textalpha. We discovered that GADD34 plays an essential role in autophagy by tuning translation during starvation, thus enabling lysosomal biogenesis and a sustained autophagic flux. Hence, the TFEB-GADD34 axis integrates the mTORC1 and ISR pathways in response to starvation.

INTRODUCTION

Amino acid starvation leads to repression of cap-dependent translation through the integrated stress response (ISR) pathway, thus decreasing global protein synthesis in the cell (fig. S1, A and B). Starvation also inhibits the mammalian target of rapamycin complex 1 (mTORC1), thus initiating autophagy and triggering a transcriptional program required for lysosomal biogenesis and a sustained autophagic flux (1). Translation of the starvation-induced transcriptional program, however, does require protein synthesis, thus conflicting with the repression of general translation. We set to investigate how this conflict is resolved.

Activation of the ISR results in phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 (eIF2\textalpha) by the GCN2 kinase. This event impairs cap-dependent translation initiation but favors selective translation of a subset of stress-responsive mRNAs containing inhibitory upstream open reading frames (uORFs), such as the transcription factor ATF4, which drives expression of essential autophagy genes such as LC3 and Atg5 (2). However, of the 513 genes annotated to lysosomal and autophagic processes according to the literature (3), only 65 (12.7%) contain multiple uORFs, and 21 (4.1%) an intra ribosomal entry sequence (4, 5), suggesting that a different mechanism must be at play.

Results

GADD34 is an early and direct TFEB target

We analyzed the global transcriptional response to increasing concentrations of TFEB upon its overexpression by means of a tetracycline-responsive promoter (Fig. 1, A to C, fig. S2, table S1, and Methods) and concomitantly performed genome-wide TFEB chomatin immunoprecipitation followed by sequencing (ChIP-seq) (Fig. 1D and table S2).

TFEB direct targets should be expressed in response to its induction in a dose-dependent manner and should have a TFEB binding site in their promoter. A total of 557 genes satisfied both conditions (Fig. 1, E to G), as they were both positively coexpressed with TFEB [Pearson correlation coefficient, false discovery rate (FDR) < 10%] and contained TFEB ChIP-seq binding sites within ±2.5 Kb from their transcription start site (table S1). Bioinformatics analysis of their binding sites revealed the previously reported CLEAR motif (Fig. 1F). Gene Ontology Enrichment Analysis (table S3) (7) of the 557 direct targets highlighted “canonical” TFEB-regulated pathways, including endolysosomal genes (e.g., AP1G1, ATP6V0D1, ATPV1F, ATP6V1H, CD68, CLN3, CTSK, GNS, LAMP1, M6PR, NAGLU, NEU1, PSAP, RRAGC, and RRAGD) and autophagic genes (ATG14, GABARAPL2, LANCL2, PLIN2, RAB1A, SQSTM1, and TP53INP2). Genes classified as “endoplasmic reticulum (ER) stress related” were also significantly enriched, including the protein phosphatase 1 (PP1)
phosphatase regulator PPP1R15A (aka GADD34), ranked as the third most correlated as shown in Fig. 1H, in addition to DDIT3 (aka CHOP, ranked 52nd) and PPP1R15B (aka CREP, ranked 384th). GADD34 undergoes selective translation upon eIF2α phosphorylation, and it is required for the activity of PP1 phosphatase that dephosphorylates eIF2α (8). Its role in the unfolded protein response (UPR) is well established: Upon eIF2α phosphorylation, GADD34 is translated and acts to terminate the response either by restoring protein synthesis, if the ER stress is resolved, or by sensitizing cells to apoptosis, if not (9). To exclude any role of ER stress in our experimental condition, that is, TFEB overexpression in nutrient-rich medium, we verified the absence of canonical markers of the UPR, i.e., no increase in Bip levels, no alternative splicing of XBP, and no transcription of ER-associated degradation (ERAD) genes (fig. S3). We next showed that the endogenous TFEB regulates GADD34 expression early during starvation. GADD34 transcript and protein levels increased quickly in response to amino acid deprivation in a TFEB-dependent manner, as concomitant knockdown (KD) of TFEB and TFE3 strongly attenuated GADD34 expression (Fig. 1I and fig. S4, C to F), while TFEB overexpression increased it (fig. S4, A and B). This is in contrast with the delayed activation of GADD34 observed after prolonged ER stress (16 hours) (9–11).

**Fig. 1. Identification of TFEB direct targets following TFEB overexpression.** (A) Characterization of inducible expression system in human embryonic kidney (HEK) 293 cells with genomic integration of the expression cassette in fig. S2. Exogenous TFEB-FLAG and tTA transactivator levels measured at the indicated time points by quantitative real-time polymerase chain reaction (qRT-PCR). (B) Quantification by densitometry of (C) immunoblotting assay (WB) of TFEB-FLAG in nuclear and cytoplasmic fractions at the indicated time points expressed as fold change relative to time 0. (D) Distribution of TFEB binding sites detected by ChIP-seq relative to the transcription start site (TSS). (E) Selection of 557 bona fide TFEB direct targets and (F) de novo motif finding in their proximal promoter revealing the CLEAR binding site. (G) Expression levels of the 557 TFEB direct targets at increasing TFEB-FLAG levels at the indicated time points. Genes are ordered according to their correlation with TFEB-FLAG expression. (H) Scatter plot of GADD34 versus TFEB-FLAG expression levels. (I) qRT-PCR of the indicated gene during amino acid deprivation in untreated cells versus TFEB-FLAG expression levels. (J) Immunoblotting assay (WB) of TFEB-FLAG in nuclear and cytoplasmic fractions at the indicated time points expressed as fold change relative to time 0. (K) Expression levels of TFEB and its direct targets at increasing TFEB-FLAG levels at the indicated time points. Genes are ordered according to their correlation with TFEB-FLAG expression. **GADD34 is required to maintain autophagic flux** We thus hypothesized that GADD34 may be necessary to initiate response to starvation by enabling translation of starvation-induced genes including lysosomal and autophagic components, under conditions (i.e., starvation) that would otherwise be not permissive for de novo protein synthesis. We assessed the impact of genetically ablating GADD34 on the autophagic flux. We thus first evaluated the number of LC3-positive structures in wild-type (WT) and GADD34 knockout (KO) cells (Fig. 2A) (12). GADD34 KO cells showed significantly more LC3-positive structures than WT cells both in growth medium and following amino acid deprivation (Fig. 2B). This effect may be explained by either an increase in autophagosome biogenesis or a decrease in autophagic flux: To distinguish between these possibilities, we treated cells with bafilomycin A1, an inhibitor of lysosome function that induces the accumulation of undigested autophagosomes—the higher the accumulation of autophagosomes induced by bafilomycin A1 treatment, the higher the rate of autophagic flux. While bafilomycin A1 induced a marked increase in LC3-positive structures in WT cells (Fig. 2, A and C), its effect was either dampened (basal and 6 hours in amino acid starvation) or absent (20 hours in amino acid starvation) in GADD34 KO cells (Fig. 2, A and C), indicating an impairment of autophagic flux in these cells.
Fig. 2. GADD34 activity is required for sustained autophagic flux in starved cells. (A) Cells lacking GADD34 have an impaired autophagy flux. WT cells or knocked out for GADD34 (GADD34 KO) in growth medium or under amino acid starvation [Hanks’ balanced salt solution (HBSS)] with or without bafilomycin A1 treatment (BafA1, 100 nM) and immunostained for LC3. Scale bar, 10 μm. (B) LC3 puncta for untreated WT and GADD34 KO cells; means ± SD. n = 150 cells per condition from three experiments; GADD34 KO cells have more LC3 puncta than WT cells (P = 1.96 × 10−12, two-way ANOVA after post hoc correction). (C and D) LC3 puncta for WT and GADD34 KO cells with BafA1 in (C), or with 50 μM salubrinal in (D), or BafA1 + salubrinal in fig. S5. P values refer to t tests between salubrinal-treated and untreated cells, or between salubrinal + Baf1−treated cells and Baf1−treated cells. (E) Western blot (WB) of Chinese hamster ovary (CHO)–WT cells following amino acid deprivation alone (CTRL), or with 100 nM BafA1, or with 50 μM salubrinal, or with BafA1 + salubrinal. (F) Densitometry of p62 and LC3-II. Means ± SD. n = 2 independent experiments; P values refer to two-way ANOVA after post hoc correction. (G) WB of GADD34 KO cells treated as in (E). (H) Densitometry of WBs. Means ± SD. n = 2 independent experiments. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. The BafA1−dependent and salubrinal-dependent increase are highlighted in orange and red, respectively.
To evaluate whether GADD34’s role in autophagic flux was indeed mediated by its ability to promote dephosphorylation of p-eIF2α, we used salubrinal, an inhibitor of eIF2α phosphatase enzymes including GADD34 (13). We first assessed the specificity of salubrinal in this context by counting LC3-positive structures in WT and GADD34 KO cells treated either with salubrinal alone or in combination with bafilomycin A1 during starvation (Fig. 2D and fig. S5). Salubrinal increased the number of LC3-positive structures in WT cells as expected, with no substantial additional effect following cotreatment with bafilomycin A1 (Fig. 2D and fig. S5). Salubrinal alone, or in combination with bafilomycin, was ineffective in GADD34 KO cells, thus indicating that salubrinal’s action on autophagy is mediated by GADD34 inhibition (Fig. 2D and fig. S5).

To further assess the autophagic flux, we also measured p62 and LC3II protein levels in WT (Fig. 2, E and F) and GADD34 KO cells (Fig. 2, G and H) during starvation in the presence of either bafilomycin A1 or salubrinal, or a combination of both drugs. In agreement with the results of the immunofluorescence studies, GADD34 KO cells exhibited higher levels of p62 and LC3II (Fig. 2, E to H). Salubrinal or bafilomycin A1 significantly increased p62 and LC3II levels in WT cells, while their combination did not induce a significant effect when compared with salubrinal alone (Fig. 2, E and F). In contrast, neither salubrinal nor bafilomycin induced significant effects in GADD34 KO cells (Fig. 2, G and H). Together, these data further support a key role of GADD34 in sustaining the autophagic flux in starved cells.

We additionally confirmed the role of GADD34 in sustaining autophagy flux also in HeLa cells by monitoring p62 and LC3II protein levels during amino acid starvation following salubrinal and/or bafilomycin A1 treatment (fig. S6, A and B) and GADD34 depletion by small interfering RNA (siRNA) (fig. S6, C and D).

Last, we measured p-eIF2α levels during amino acid starvation. As shown in fig. S7 (A and B), the levels of p-eIF2α increased upon amino acid deprivation, as previously reported (10), and this increase was amplified by salubrinal. TFEB overexpression significantly decreased p-eIF2α (in a salubrinal-sensitive fashion) in agreement with its role in increasing GADD34 expression and, thus, PP1 phosphatase activity (fig. S7, A and B), while TFEB/TFE3 or GADD34 depletion had the opposite effect (fig. S7, C and D). These results showed that increased levels of GADD34 during starvation are required to prevent excessive eIF2α phosphorylation (p-eIF2α).

Overall, our data demonstrate that GADD34 inhibition causes excessive phosphorylation of eIF2α leading to a reduced autophagic flux. Phosphorylation of eIF2α is required to promote and sustain autophagy during starvation, as cells with a permanently unphosphorylated eIF2α mutant (S51A) exhibited a reduced autophagic response to starvation (fig. S8, A to D). Thus, both excessive phosphorylation and a lack thereof are detrimental to the autophagic flux, and both impair the cell ability to survive prolonged starvation (fig. S8E).

**GADD34 is required for lysosome biogenesis in response to starvation**

We next assessed the role of GADD34 on TFEB-mediated lysosome biogenesis, a process necessary to sustain the autophagic flux during starvation. To this end, we developed an assay in a cell model of Fabry disease (14), a lysosomal storage disorder due to the loss of function of galactosidase alpha (encoded by the GLA gene): the GLA-KO cells. As depicted in Fig. 3A, "newly formed" lysosomes can be distinguished from "old" ones in GLA-KO cells as they are smaller and devoid of storage material [Gb3, stained with Shiga Toxin (15)]. Following amino acid deprivation, the overall number of lysosomes in GLA-KO cells increased significantly (Fig. 3, B and C), and, specifically, of "new" lysosomes as indicated by the increase in small storage-free lysosomes (Fig. 3D, red bars). Salubrinal treatment completely abolished the effect of starvation on lysosomal biogenesis as evidenced by the lack of change in total lysosomal number (Fig. 3, B and C) and specifically of small new lysosomes (Fig. 3D, red bars). To further confirm these results, we analyzed lysosomal biogenesis also in WT and GADD34 KO cells by counting the total number of lysosomes under growth and starvation conditions (Fig. 3, E and F). Only in WT cells, but not in GADD34 KO cells, did starvation induce an increase in lysosome number. Together, these results support the role of GADD34 as an enabler of lysosomal biogenesis by dephosphorylating p-eIF2α.

**GADD34 activity is required to translate the starvation-induced transcriptional program**

We next asked whether GADD34 is necessary to enable translation of transcripts during starvation. To this end, we performed high-resolution quantitative proteomics in control conditions (growth medium) and following amino acid deprivation [Hanks’ balanced salt solution (HBSS)] in cells with either an active or blocked (HBSS + salubrinal) GADD34 activity. By applying bio-orthogonal amino acid tagging with acid azidohomoalanine (AHA), we focused on proteins that are actively synthesized during starvation (16). As shown in fig. S1B, the pattern of labeling with AHA confirmed that de novo protein synthesis is lower in starved than in control cells. This finding is consistent with the puromycin labeling pattern following amino acid deprivation (fig. S1A).

By quantitative proteomics, we identified 3667 proteins (table S4) that were present in at least one of the three conditions (growth medium, HBSS, and HBSS + salubrinal). As shown in Fig. 4A, amino acid deprivation alone (HBSS versus growth) significantly [one-way analysis of variance (ANOVA) post hoc test, P < 0.1] changed 968 proteins (up-regulating 477 and down-regulating 491). Down-regulated proteins were enriched for highly expressed proteins related to protein anabolism such as ribosomal components, in addition to mitochondrial proteins, and included autophagy substrates (e.g., SQSTM1/p62, GABARAP2L) (Fig. 4A and table S5). These proteins belong to classes known to be preferentially degraded early in response to amino acid deprivation (17).

Up-regulated proteins belonged to classes related to protein catabolism, including lysosomal and autophagic proteins (ATP6V1H, CAT, CTSB, CTSC, CTSL, CTSL2, LANCL2, GNS, and PLIN2), endosome/multivesicular body proteins (AP1G1, CHMP1B, CHMP2B, EEA1, Rab7A, and VPS35), Golgi proteins (COPB1 and GALNT5), and the proteasome (PSMA1-5, PSMB2-6, PSMC2-5, PSMD2, PSMD11, and PSMD14) (Fig. 4B and table S6). These observations are in line with the previously reported increase in proteasome subunits and activity, and lysosomal biogenesis in response to nutrient deprivation (18, 19). Blocking GADD34 activity during starvation (HBSS + salubrinal versus HBSS) resulted in a significant (one-way ANOVA post hoc test, P < 0.1) decrease in the synthesis of the very proteins belonging to the endolysosomal system, including TFEB direct targets, as reported in Fig. 4 (A and B). On the contrary, proteins increasing the most included those degraded by autophagy (e.g., SQSTM1/p62 and GABARAPL2 in Fig. 4A), consistently with the reduced autophagic...
Regulation of GADD34, a key effector of the ISR. Our results support the model depicted in Fig. 4C. Upon nutrient deprivation, the kinase GCN2 phosphorylates eIF2α, thus reducing global protein synthesis and inducing specific translation of the transcription factor ATF4, a known activator of GADD34 transcription following ER stress (9, 10). Concomitantly, inhibition of mTORC1 induces TFEB nuclear accumulation, which in turn activates a transcriptional program to promote lysosomal biogenesis and increase autophagic flux.

DISCUSSION

Regulation of translation under starvation is a complex process that requires cross-talk between the mTOR and ISR pathways. The ISR induces eIF2α phosphorylation, thus inhibiting CAP-dependent translation and favoring translation of eIF2α-sensitive mRNAs, while acute mTOR inhibition reduces translation of selected mRNAs via LARP1- and 4E-BP–dependent mechanisms (20) and activates TFEB (1, 6). Our work demonstrates a novel and essential interplay between these two pathways during starvation involving TFEB-mediated regulation of GADD34, a key effector of the ISR. Our results support the model depicted in Fig. 4C. Upon nutrient deprivation, the kinase GCN2 phosphorylates eIF2α, thus reducing global protein synthesis and inducing specific translation of the transcription factor ATF4, a known activator of GADD34 transcription following ER stress (9, 10). Concomitantly, inhibition of mTORC1 induces TFEB nuclear accumulation, which in turn activates a transcriptional program to promote lysosomal biogenesis and increase autophagic flux.

TFEB also directly binds the ATF4 promoter, thus enhancing its expression following prolonged ER stress and starvation (11). Here, we show that to enable an effective translation of the starvation-induced transcriptional program, TFEB directly drives early expression of GADD34, which is selectively translated in the presence of phosphorylated eIF2α (21). GADD34 activity prevents excessive eIF2α phosphorylation, thus allowing translation of the starvation-induced transcriptional program to occur. This role of GADD34 in starvation has some parallels to its function in infected cells in response to double-stranded RNAs, where it is needed to allow cytokine production in the face of a general mRNA translation block to prevent viral replication (22). A link between GADD34 and autophagy has been previously reported, but it was related to mTOR inactivation [by starvation (23), by ER stress (24, 25), or by the expression of mutant huntingtin proteins (26)], and never associated with either the starvation-induced TFEB transcriptional response or a role in lysosome biogenesis, the two key findings of our work.

Previous studies have shown that eIF2α phosphorylation is necessary for protracted autophagy during starvation, but the mechanisms remain unclear (27). Here, we show that the two extremes, no phosphorylation or excessive phosphorylation of eIF2α, are both
deleterious (fig. S8E). In the first case, general protein synthesis is not reduced, thus preventing catabolism of amino acids for energy production. In the latter case, excessive phosphorylation causes an extreme reduction in protein synthesis, preventing translation of starvation-responsive genes. Our work raises the question of how a cell determines the optimal level of eIF2α phosphorylation in response to starvation. Understanding this regulatory mechanism may yield novel ways to modulate autophagic flux, which do not directly depend on the mTOR pathway, and may potentially benefit those disorders where autophagy induction is thought to be beneficial, including neurodegenerative diseases and aging.

MATERIALS AND METHODS

Cell cultures

Human embryonic kidney (HEK) 293, HEK-293FT (for lentivirus production), and HeLa (WT and GLA-KO) cells were grown at 37°C in an atmosphere of 5% CO₂ and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Thermo Fisher Scientific–Gibco). The media for HEK-293 and HeLa cells’ growth were supplemented with 10% fetal bovine serum (FBS) (catalog number 0270-098, Thermo Fisher Scientific–Gibco), 2 mM l-glutamine, and penicillin/streptomycin (100 U/ml) (catalog number 15140122, Thermo Fisher Scientific–Gibco), while the media for the HEK-293FT cells’ growth were supplemented with 10% heat-inactivated FBS (catalog number A3840001, Thermo Fisher Scientific–Gibco), 2 mM l-glutamine, 1× MEM nonessential amino acids, 1 mM sodium pyruvate, and penicillin/streptomycin (100 U/ml) (catalog number 15140122, Thermo Fisher Scientific–Gibco). Chinese hamster ovary (CHO) cells (WT, GADD34 KO, and p-eIF2α<sup>512A</sup>) were grown in Ham’s F-12 supplemented with 10% FBS (catalog number 0270-098, Thermo Fisher Scientific–Gibco), 2 mM l-glutamine, and penicillin/streptomycin (100 U/ml) (catalog number 15140122, Thermo Fisher Scientific–Gibco). When confluent, the cells were trypsinized with 0.25% trypsin/EDTA (catalog number T4049, Sigma-Aldrich) for 1 min and then plated at a density of 10<sup>4</sup> cells/cm. HBSS with calcium and magnesium (Gibco catalog number 24020117) was used as starvation medium.

Cloning strategy

The cassettes comprising the genetic circuits were implemented by using the ViraPower Promoterless Lentiviral Gateway expression system (catalog number 442050, Thermo Fisher Scientific). Entry vectors for Gateway cloning of the PFL were designed as described.
in (28) and synthesized by Geneart. The blunt-ended polymerase chain reaction (PCR) product of TFEB3xFlag sequence was amplified from pCMV_TFEB3xFlag by using the High-Fidelity Taq Phusion (catalog number F553, Fynnzimes) and then cloned into pCR-Blunt II TOPO vector by using the Zero Blunt TOPO PCR Cloning Kit (catalog number 450245, Thermo Fisher Scientific–Invitrogen). We digested both pCR-Blunt II TOPO–TFEB and pMATTA-IRES-d2EYFP, already available in the laboratory (29), with Nhe I and Eco RV restriction enzymes (NEB) to ligate TFEB in place of the d2EYFP, thus obtaining pMATTA-IRES-TFEB. To stabilize the transcripts, we kept the WPRE sequence also present in the original vector. The pMATTA-IRES-WPRE cassette was amplified by High-Fidelity Taq Phusion PCR and cloned into the pENTR directional TOPO vector (catalog number 450218, Thermo Fisher Scientific–Invitrogen) with the specific recombination sites to generate pENTR-ttA-IRES-TFEB vector. To generate the lentiviral vector containing the gene expression cassette PFF–TFEB, the reconstruction reaction was performed between the pENTR–tta-IRES-TFEB-WPRE, the pENTR59-TOPO-CMV-TET already available in the laboratory, and the pLenti/R4R2/V5-DEST (catalog number V498-10, Thermo Fisher Scientific) according to the manufacturer’s instructions. According to the manufacturer’s instructions, the lentivirus was then produced in 293FT cells as previously described (29).

Transfection
When confluent (80%), the HEK-293 cells were transfected in six-well plates with Lipofectamine LT (catalog number 15338100, Thermo Fisher Scientific–Life Technologies): 1000 ng of DNA was mixed with Opti-MEM I reduced serum medium (catalog number 3198506, Thermo Fisher Scientific) to a final volume of 100 µl followed by addition of 1 µl of PLUS reagent; 5 min later, 1.8 µl of Lipofectamine LTIX and of transfection complex was added dropwise to the seeded cells; 6 hours later, the transfection medium was replaced with fresh medium. HeLa cells were transfected in a six-well plate with TFEB–green fluorescent protein plasmid (gift from A.B.’s laboratory) with Transit-LT1 (Mirusbio LLC) according to the manufacturer’s instructions and incubated for 18 to 24 hours before fixation.

siRNA treatment
siRNA duplexes against TFE3 and TFE3 were purchased as smartpool from Dharmacon. siRNA duplexes against ATF4 and GADD34 were purchased from Sigma–Aldrich. Three siRNA duplexes were used to knock down ATF4 and GADD34. The sequences of the siRNA used are listed below:

| siRNA     | Sequence (Sense)                        | Sequence (Antisense) |
|-----------|----------------------------------------|----------------------|
| siRNA-hGADD34| 5′-GUGGUAGUAGUAGAAGAA[dT][dT]            | 3′-dAAGUGUAGAAGAAGA[dT][dT]  |
| siRNA-hGADD34| 5′-GACACUGUAGUUGCUAUA[dT][dT]            | 3′-dUACCUAGUAGCUGAAG[dT][dT]  |
| siRNA-hATF4 | 5′-GUGGAAACUGUAGAAGG[dT][dT]             | 3′-dCUCUCGAGCAAGAAG[dT][dT]  |

HeLa cells were transfected with siRNAs for 96 hours using Oligofectamine (Thermo Fisher Scientific) according to the manufacturer’s instructions. The siRNA duplexes were used at 25 pmol for TFE3, TFE3, ATF4, and GADD34. Mock-treated or nontargeting siRNA-treated HeLa cells are referred to as controls (CTRL).

Quantitative real-time PCR
Quantitative real-time PCR (qRT–PCR) was carried out with the LightCycler 480 SYBR Green I mix (Roche) using the LightCycler 480 II detection system (Roche) with the following conditions: 95°C, 5 min; (95°C, 10 s; 60°C, 10 s; 72°C, 15 s) × 20 cycles. For expression studies, the qRT–PCR results were normalized against an internal control (HPRT). The primers used in this study are the following:

**Lentivirus production and monoclonal cell population**
Lentiviral particles carrying PFF–TFEB construct were produced in HEK-293FT cells with ViraPower Lentiviral Expression System (catalog number K497500, Thermo Fisher Scientific) according to the manufacturer’s instructions. Subconfluent HEK-293 cells were transduced with viral particles, and stable clones were selected with Blasticidin S (4 µg/ml; catalog number R21001, Thermo Fisher Scientific). A monoclonal cell population stably expressing the PFF–TFEB gene expression cassette (HEK_PFF–TFEB) was isolated with serial dilutions and used to perform farther time series experiment.

**Compounds**
Tetracycline (catalog number T7660) and doxycycline (catalog number D3447) were purchased from Sigma–Aldrich and used at the concentrations of 100 ng/ml and 1 µg/ml, respectively, in the culture media. Salubrinal (catalog number SML0951) and bafilomycin A1 (catalog number B1793) were purchased from Sigma–Aldrich.

**Antibodies**
ANTI-FLAG (M2) (catalog number F1804) was used at 1:1000 dilution. Anti-β-actin was purchased from Sigma–Aldrich (catalog number A2228) and was used at 1:5000 dilution. Anti–GAPDH (glyceraldehyde-3-phosphate dehydrogenase)–mouse purchased from Santa Cruz Biotechnology (catalog number sc-32233) was used at 1:5000 dilution. Anti-histone H3 antibody is a rabbit polyclonal antibody purchased from Merck Millipore (catalog number 07-555) and was used at dilution 1:1000. Anti–epidermal growth factor receptor (EGFR) (catalog number NC-03, Santa Cruz Biotechnology, Santa Cruz, CA) is a polyclonal antibody epitope mapping to the C-terminal of human EGFR and was used at 1:1000 dilution. Anti-BIP (catalog number 31-77), anti-p-eIF2α (catalog number 3295), and anti-eIF2α (catalog number 5324) were purchased from Cell Signaling Technology and used at 1:1000 dilution. The bead-conjugated antibody ANTI-FLAG M2 Affinity Gel
Puromycin incorporation assay

HeLa cells (~80% confluent) in normal growth conditions (full medium) and amino acids starved (HBSS, Gibco catalog number 14025092) for 1 and 3 hours were incubated with puromycin (1 g/ml) for 1 and 3 hours were incubated with puromycin (1 g/ml) for the quantification of the specific band, located in a signal-free area of the membrane. In cases of membranes with variable background, background subtraction was performed for each lane independently.

LC-MS/MS analysis and protein quantification

Five microliters of the obtained peptide solution was injected on a nanoACQUITY chromatographic system (Waters, Milford, MA, USA) and then analyzed with a TripleTOF 5600+ mass spectrometer equipped with a NanoSpray III ion source (SCIEX, Ontario, Canada). The peptides were first loaded on a trapping column (180 µm by 20 mm Acquity C18 column), desalted for 4 min at 4 µl/min at 1% ACN for 10 min and then moved to a Picofrit column (C18 column, 20 mm Acquity C18 column), desalted for 4 min at 4 µl/min at 1% ACN + 0.1% FA, and then moved to a Picofrit column (C18 column, 75 µm by 25 cm, from New Objective Inc., Woburn, MA, USA). A 2-hour linear gradient from 3 to 45% ACN in H₂O, both added with 0.1% FA, was used to elute peptides at 300 nl/min. The column was then washed with 90% ACN for 10 min and the reequilibrated to 3% ACN for 18 min. Analysis was then performed in positive ion mode with the following parameters: ion spray voltage, 2500 V; spray gas 1, 10; curtain gas, 30; declustering potential, 80 V; and source temperature, 90°C. Spectra were acquired in data-independent acquisition mode following the SWATH protocol for label-free proteomics (31). The mass/charge ratio (m/z) range of precursor ions went from 400 to 1250, with a variable window width from 7 to 50 Da. The instrument first acquired a full range scan of 250 ms, and then 100 consecutive SWATH experiments, each lasting 25 ms, were performed within the 100 to 1500 m/z range. The obtained SWATH spectra were imported in PeakView software and then searched against the PanHuman ion library (32). For the protein quantification, the following settings were used: use only nonshared and nonmodified peptides, minimum peptide confidence 90%, 50 ppm maximum mass tolerance, 30 min maximum RT tolerance, and six MRM transitions per peptide. A total of 3667 proteins were quantified, and the corresponding raw data files were imported in MarkerView software to perform a normalization using the most likely ratio method (33). Differentially expressed proteins across conditions were identified using one-way ANOVA, and post hoc test was performed using the function aov and TukeyHSD in R statistical environment.
Inducible HEK_PFL-TFEB monoclonal cells (2.5 × 10^5) were plated for 3 days in a 24-well plate, each well containing 1 ml of medium supplemented with tetracycline (100 ng/ml), the antibiotic was removed from the culture medium at time 0 hour to induce TFEB expression, and samples were collected every 6 hours up to 90 hours for RNA extraction. Total RNA extraction was performed using the Qiagen RNeasy Kit (catalog number 74106, Qiagen) according to the manufacturer’s instructions. When necessary, retrotranscription of 1 μg of the total RNA extracted was performed using the QuantiTect Reverse Transcription Kit (catalog number 205313, Qiagen) according to the manufacturer’s instructions. qRT-PCRs were set up in triplicate with Applied Biosystems SYBR Green. One to five nanograms of purified DNA was amplified on an Applied Biosystems 7500 Fast Real-time PCR system for 40 cycles. PCR duplicates, multimapping reads, and reads having a mapping quality (MAPQ) score smaller than 5 were discarded using SAM tools (39). Peak calling was performed by using MACS2 (40), comparing each sample in which TFEB was overexpressed with the corresponding input sample. MACS2 was used with default parameters and bin width = 200. Regions identified by MACS2 as bound by TFEB (P < 5 × 10^-3) in at least two conditions (i.e., time points) and overlapping for more than 10 bp were merged together. Binding regions were then annotated using the ChiPseeker package (41) in R statistical environment.

Identification of TFEB target genes
TFEB-FLAG expression measured across the 16 time points was correlated with all the other 17,488 genes measured from RNA sequencing (RNA-seq) experiment using the function cor.test of R statistical environment. P values were then corrected for FDR using Benjamin and Hochberg correction of p.adjust function of R statistical environment. After correction for FDR, only genes with an FDR less than 10% were considered correlated with TFEB expression. Last, direct targets of TFEB were defined as genes positively correlated with TFEB expression and having a binding site of TFEB in the ±2500 bp of their promoter region identified as described above.
Identification of TFEB consensus binding sequence

TFEB consensus binding sequence was identified using the Weeder tool 2.0 (42). Weeder tool was run using human motifs (-O HS parameter) in single-strand mode (-ss parameter) and discarding from the output those motifs that were too similar to other motifs already found (-sim 0.1 parameter). Ten cycles of expectation maximization (EM) instead of only one as in the default run mode were performed to obtain more robust results (-en 10 parameter). The tool was run using as input the sequences of the identified binding regions associated with the 557 direct targets of TFEB identified in this study. The estimated position weight matrix of the TFEB consensus binding sequence was identified using the Weeder tool. The estimated position weight matrix of the TFEB consensus bind- using as input the sequences of the identified binding regions asso- to obtain more robust results (-parameter). The tool was run −em 10 −sim 0.1 found (-parameter). Ten cycles of expectation maximization

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