Acetyltransferase GCN5 regulates autophagy and lysosome biogenesis by targeting TFEB

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Abstract

Accumulating evidence highlights the role of histone acetyltransferase GCN5 in the regulation of cell metabolism in metazoans. Here, we report that GCN5 is a negative regulator of autophagy, a lysosome-dependent catabolic mechanism. In animal cells and Drosophila, GCN5 inhibits the biogenesis of autophagosomes and lysosomes by targeting TFEB, the master transcription factor for autophagy- and lysosome-related gene expression. We show that GCN5 is a specific TFEB acetyltransferase, and acetylation by GCN5 results in the decrease in TFEB transcriptional activity. Induction of autophagy inactivates GCN5, accompanied by reduced TFEB acetylation and increased lysosome formation. We further demonstrate that acetylation at K274 and K279 disrupts the dimerization of TFEB and the binding of TFEB to its target gene promoters. In a Tau-based neurodegenerative Drosophila model, deletion of dGcn5 improves the clearance of Tau protein aggregates and ameliorates the neurodegenerative phenotypes. Together, our results reveal GCN5 as a novel conserved TFEB regulator, and the regulatory mechanisms may be involved in autophagy- and lysosome-related physiological and pathological processes.

Keywords acetylation; autophagy; GCN5; lysosome; TFEB

Subject Categories Autophagy & Cell Death; Post-translational Modifications & Proteolysis

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Introduction

Autophagy is a major cellular turnover pathway whereby intracellular contents are delivered by double-membraned autophagosomes to lysosomes for degradation. While the basal constitutive autophagy serves to clear undesired cellular substances, cells initiate adaptive autophagy in response to intracellular and extracellular cues such as nutrient limitation, DNA damage, and oxidative stress [1]. Therefore, autophagy plays a dual role of cell metabolism regulation and intracellular quality control, and increasing evidence suggests that dysfunctional autophagy is involved in many pathological processes, such as cancer and neurodegenerative disease [2].

The implementation of autophagy and sustained autophagy flux requires adequate lysosomal activity. Therefore, autophagy is intimately associated with the biogenesis and activation of lysosomes. The role of transcription factor EB (TFEB) is important in this process, on account of the fact that it simultaneously regulates the expression of genes for autophagosome formation and lysosome production [3,4]. As a member of the MIT/TFE family of transcription factors, the activity of TFEB is mainly regulated by mechanistic target of rapamycin (mTOR), which determines the subcellular localizations of TFEB. Phosphorylation of TFEB by mTOR binds TFEB to 14-3-3 protein and retains it in the cytoplasm [5,6], while inactivation of mTOR and/or activation of the phosphatase calcineurin results in dephosphorylated TFEB, which enters nucleus [7]. In addition to mTOR, protein kinase C, glycogen synthase kinase, and Rac-alpha serine/threonine protein kinase can also phosphorylate TFEB and regulate its translocation from cytoplasm to nucleus [8–10]. Interestingly, recent studies have shown that the transcriptional activity of TFEB is also regulated by its acetylation, and the deacetylation of TFEB significantly improves autophagy and lysosomal function [11]. This suggests that the intracellular acetylation/deacetylation system can not only directly target autophagy-related proteins [12–15], but also directly regulate autophagy at the transcriptional level. Although acetyl-coenzyme A acetyltransferase 1 (ACAT1) and the histone deacetylases SIRT1 and HDAC2 have been reported to affect the acetylation of TFEB [11,16], the molecular mechanism by which acetylation regulates TFEB activity remains unclear.

General control non-repressed protein 5 (GCN5) was initially identified as a histone acetyltransferase in Tetrahymena [17,18]. Like other acetyltransferases, besides targeting core histones, GCN5 also exerts its gene regulatory function through acetylation of sequence-specific transcription factors [19–22]. This enables it to participate in a wide range of cellular processes, including cell proliferation, differentiation, telomere maintenance, and DNA
damage repair [20,23–25]. Intriguingly, recent studies have shown that GCN5 is an important regulator of cell metabolism. GCN5 acetylates and inactivates peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), thereby regulating the expression of many genes required by various cellular metabolic pathways, including fatty acid oxidation and gluconeogenesis [19,26]. In addition to being activated by its coenzyme acetyl-CoA, the acetyltransferase activity of GCN5 can also be up-regulated by the essential amino acid methionine and insulin–GSK3β signals [27–29]. Animal models showed that the expression of GCN5 is increased by a high-fat diet and decreased by fasting [22,30]. All these results indicate a role for GCN5 in cell energy homeostasis.

However, the function of GCN5 in autophagy, a key process of cell catabolism, is still unknown.

In this study, we have determined that GCN5 functions as an inhibitor of autophagy and lysosomal biogenesis in mammalian cells and Drosophila. Mechanistically, GCN5 acetylates TFEB, which disturbs the dimerization of TFEB and subsequently the binding of TFEB to its target gene promoters. Silencing GCN5 in a Tau-based Drosophila neurodegeneration model ameliorates the neurodegenerative phenotypes by facilitating the elimination of Tau protein aggregates.

Results

GCN5 negatively regulates autophagy

To assess the potential role of GCN5 in the regulation of autophagy, we generated GCN5 knockout (GCN5 KO) HeLa and HEK293 cells lines using the CRISPR/Cas9 system. In these cells, an increase in the number of LC3 puncta and the protein level of LC3-II was detected (Figs 1A, B and E, and EV1A–C). The same results were obtained from cells treated with a specific GCN5 inhibitor, α-methylene-γ-butyrolactone 3 (MB-3) (Figs 1C and EV1D and E). Transfection in GCN5 KO cells of wild-type (WT) GCN5 but not the acetyltransferase-defective GCN5-E575Q mutant [31,32] eliminated the increase in LC3 puncta (Fig 1D and E). Furthermore, overexpression of GFP-GCN5 reduced LC3 puncta and LC3-II in WT HeLa cells that show a high level of basal autophagy (Figs 1F–H and EV1F). These data thus suggest an inhibitory effect of GCN5 on autophagosome formation. To evaluate autophagic degradation, we checked the expression of SQSTM1/p62, an autophagy adaptor that is degraded by autophagy. Unexpectedly, in GCN5 KO cells, compared with WT cells, we detected an increase in the level of p62 mRNA, but no significant change in the level of p62 protein (Fig EV1G and H), which suggests that GCN5 inhibited p62 transcription. We then checked the degradation of exogenous GFP-p62, whose transcription is TFEB-independent, in HEK293 cells stably expressing GFP-p62. GCN5 knockdown had a significant effect on this localization (Fig 1K). However, knocking down GCN5 significantly promoted the formation of mCherry-Atg8a puncta in starved Drosophila larvae, while overexpression of dGcn5 attenuated the formation of puncta (Fig 1K). Taken together, these data suggest that GCN5 is an inhibitor of autophagy.

GCN5 inhibits lysosomal biogenesis

In GCN5 KO cells, we also observed an increase in the number of lysosomes indicated by lysosome-associated membrane glycoprotein 1 (LAMP1)-positive and LysoTracker-labeled punctate structures (Figs 2A, B and E, and EV2A), accompanied by an increase in the expression of lysosomal proteins including LAMP1 and mature cathepsin D (CTSD) (Figs 2C and EV2B and C). Transfection in the cells of WT GCN5 but not the GCN5-E575Q abolished the increase in lysosome number (Fig 2D and E). In addition, the activity of the lysosomal enzyme β-hexosaminidase increased significantly in these cells (Fig 2F). To further verify the increase in lysosomal activity in

Figure 1. GCN5 negatively regulates autophagy.

A LC3 punctum showing LC3-II formation in WT and GCN5 KO HeLa cells (Scale bars, 10 μm).
B Immunoblot showing LC3-II formation in WT or GCN5 KO or MB-3-treated HeLa cells in the presence or absence of the lysosome inhibitor Baf.
C Formation of LC3 puncta in GCN5 KO HeLa cells overexpressing Myc-tagged GCN5 or GCN5-E575Q (Scale bars, 10 μm).
D Quantification of LC3 puncta in cells shown in (A) and (D). The cells were treated with or without Baf (graph represents data from three independent experiments with ≥ 30 cells per condition; mean ± SEM, ***P < 0.001, Student’s t-test).
E Formation of LC3 puncta in HeLa cells overexpressing GFP-GCN5 with or without Baf treatment (graph represents data from three independent experiments with ≥ 30 cells per condition; mean ± SEM, *P < 0.05, ***P < 0.001, Student’s t-test; Scale bar, 10 μm).
F LC3-II formation in GFP-GCN5-overexpressing HeLa cells.
G GFP-p62 levels in HEK293 cells stably expressing GFP-p62. The cells were cultured with GCN5 siRNA with or without CQ.
H PDLIM1 and IFT20 protein levels in GCN5 KO HEK293 cells with or without transfection of GFP-GCN5 and addition of CQ.
I Representative images of mCherry-Atg8a (red) and DAPI (blue) in Drosophila larval fat body in which dGcn5 is overexpressed (OE) or silenced (KD) using the pan-fat body driver (cg-GAL4). Drosophila (cg-GAL4/F+) was used as the control (graph represents data from three independent experiments with ≥ 30 cells per condition; mean ± SEM, *P < 0.05, ***P < 0.001, Student’s t-test; Scale bars, 10 μm).

Source data are available online for this figure.
the cells, we analyzed the processing of epidermal growth factor receptor (EGFR). The absence of GCN5 obviously accelerated EGFR degradation in EGF-stimulated cells (Figs 2G and EV2D). Finally, we assessed the role of GCN5 in lysosomal biogenesis in Drosophila. Under feeding conditions, consistent with previous observations [36], LysoTracker marked a few spots in the fat body of Drosophila larvae. The deletion of dGCN5 significantly increased the abundance of LysoTracker-positive punctate structures (Fig 2H). In addition, deletion of dGCN5 further promoted the starvation-stimulated formation of LysoTracker puncta, while overexpression of dGCN5 reduced their formation (Fig 2H). Together, these results suggest that GCN5 is an inhibitor of lysosomal biogenesis.
Figure 2. GCN5 inhibits lysosomal biogenesis.
A LAMP1 puncta (green) and DAPI (blue) in WT and GCN5 KO HEK293 cells (Scale bars, 10 μm).
B Fluorescence-activated cell sorting analysis of WT and GCN5 KO HEK293 cells stained with LysoTracker. Fluorescence intensity of 10,000 cells per sample was measured.
C Immunoblot showing lysosomal protein levels in three independent clones of GCN5 KO HEK293 cells. CTSD HC, cathepsin D heavy chain.
D LAMP1 puncta in GCN5 KO HEK293 cells overexpressing Myc-tagged GCN5 or GCN5-E575Q (Scale bars, 10 μm).
E Quantification of LAMP1 puncta in (A) and (D) (graph represents data from three independent experiments with ≥30 cells per condition; mean ± SEM; ***P < 0.001, Student’s t-test).
F Hexosaminidase activity in GCN5 KO HEK293 cells (mean ± SEM; n = 3 independent experiments; ***P < 0.001, Student’s t-test).
G Degradation of EGFR in WT and GCN5 KO HEK293 cells in the presence or absence of CQ.
H Representative images of LysoTracker staining (red) and DAPI (blue) in Drosophila larval fat body in which dGcn5 is overexpressed (OE) or silenced (KD). Drosophila (cg-GAL4+/+) was used as the control (graph represents data from three independent experiments with ≥30 cells per condition; mean ± SEM; *P < 0.05, **P < 0.01, Student’s t-test; scale bars, 10 μm).
Source data are available online for this figure.
GCN5 acetylates TFEB at K116, K274, and K279

The effect of GCN5 on autophagosome formation, lysosomal biogenesis, and p62 transcription suggests a potential role of GCN5 in regulating TFEB. To clarify this, we first generated GCN5 and TFEB double knockout cells based on the GCN5 KO cell line and found that deletion of TFEB completely eliminated the increase in LC3-puncta, LC3-II level, and lysosome number induced by GCN5 KO.
and K450 (Fig EV3I). Using gene overexpression and gene knockdown, Gcn5 has acetyltransferase activity toward dMitf of autophagy-related transcriptional activity of TFEB, we first examined the expression of TFEB target genes. Using a luciferase reporter carrying TFEB-binding sites (CLEAR element) [3], we found that GCN5 KO cells (Fig 3I) and TFEB-WT-transfected cells (Fig 4E) showed higher luciferase activity than WT cells and TFEB-WT-transfected cells, respectively, while TFEB depletion prevented the effect of GCN5 KO (Fig 4D). Together, these results suggested that GCN5-mediated acetylation inhibits the transcriptional activity of TFEB.

Acetylation disrupts the dimerization and DNA-binding activity of TFEB

To investigate the molecular mechanism underlying the acetylation-mediated inactivation of TFEB, we checked the effect of GCN5 on the intracellular localization of TFEB, which is directly related to TFEB transcriptional activity and is mainly regulated by mTORC1. We noted that deletion of GCN5 affected neither the dominant cytoplasmic distribution of TFEB under basal conditions nor the nuclear accumulation of TFEB after treatment with the mTOR inhibitor Torin1 (Figs 5A and EV4A). In addition, deletion or inhibition of GCN5 showed no effect on mTORC1 activity, as indicated by unchanged the phosphorylation of mTORC1 substrate 4E-BP1 (Fig EV4B). Next, we used the TFEB-S211A mutant, which has prominent nuclear localization [6], as a positive control to explore the function of acetylation on TFEB localization. Both the GCN5 acetylation-defective TFEB-3KR and the acetylation-mimetic TFEB-3KQ, in which the three lysines at the acetylation sites by GCN5 were changed to glutamine, remained in the cytoplasm and transferred normally into the nucleus when the cells were treated with Torin1 (Figs 5B and EV4C). These results suggest that GCN5-dependent acetylation does not affect the intracellular distribution of TFEB. These data therefore support the view that GCN5, which is predominantly localized in the nucleus [19,38], regulates intracellular TFEB activity mainly by targeting the nuclear pool of TFEB. In fact, with cell fractionation, we found both in TFEB-Flag-expressing cells and in WT cells that a small proportion of TFEB-Flag and endogenous TFEB was present in the nucleus even in basal fed culture, which did not depend on their acetylation (Fig EV4D–F). In addition, compared with cytoplasmic TFEB, nuclear TFEB showed a much higher acetylation level, which was strongly inhibited by the treatment of cells with GCN5 inhibitor MB-3 (Fig EV4G). We then performed a chromatin immunoprecipitation (ChIP) assay to detect a potential effect of GCN5-mediated acetylation on the binding of TFEB to its target gene promoters. Intriguingly, compared with WT TFEB, TFEB-3KR showed stronger binding to the promoters of the TFEB target genes CLCN7, GLA, and CTSD in cells, whereas TFEB-3KQ did the opposite (Fig 5C). This was mainly attributed to deacetylation at K274 and K279, because the TFEB-K274R/K279R mutant exhibited similar binding capacity to TFEB-3KR, while the binding of the TFEB-K116R mutant was similar to that of TFEB-WT (Fig 5C). We further carried out in vitro electrophoretic mobility shift assays (EMSA) in which purified recombinant TFEB or the TFEB mutants were incubated with a DNA fragment containing the TFEB-binding region within the GLA promoter. We found that incubation with TFEB-WT or TFEB-K116Q, but not TFEB-3KQ or TFEB-K274Q/K279Q, resulted in a strong up-shift of the GLA promoter DNA (Fig 5D). These results therefore indicated that acetylation at K274 and K279 reduces the binding of TFEB to its target gene promoters, which was consistent with the ChIP assay results. However, it is noteworthy that TFEB-3KQ or TFEB-K274Q/K279Q incubation, like TFEB-WT or TFEB-K116Q incubation, reduced the activity of the promoter of TFEB target genes. Using a luciferase reporter carrying TFEB-binding sites (CLEAR element) [3], we found that GCN5 KO cells (Fig 4D) and TFEB-3KR-overexpressing cells

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level of free GLA promoter DNA (Fig 5D). Meanwhile, incubation with TFEB-3KQ or TFEB-K274Q/K279Q caused a smeared up-shift of the GLA promoter DNA (Fig 5D). These observations implied that acetylation at K274/K279 may influence the stability of TFEB-DNA binding instead of the constitutive capacity of TFEB for DNA association.

It has been reported that high-affinity DNA binding of TFEB requires TFEB to form homodimers or heterodimers with other members of the MiT/TFE family [39]. Both K274 and K279 are located in helix 2 of the bHLH domain, which is conserved in all the MiT/TFE family proteins and necessary for the formation of homodimers or heterodimers [40,41]. Structural modeling predicts that the substitution of K-to-Q but not K-to-R at K274 and K279 may cause steric hindrance between TFEB and its dimeric partner (Fig 5E). This suggests that acetylation of these residues may affect TFEB dimerization. To test this, we performed co-immunoprecipitation analysis in cells co-transfected with Myc-labeled and Flag-labeled TFEB or the acetylation-mimetic TFEB mutants. Interestingly, co-precipitations occurred between TFEB-WT, TFEB-WT, and TFEB-K116Q, but not between TFEB-WT and TFEB-K274Q/K279Q (Figs 5F and EV4H). TFEB-K274Q/K279Q also showed weaker interaction with MITF and TFE3 (Figs 5G and H, and EV4I and J). Further, we performed in vitro glutaraldehyde cross-linking analysis of purified recombinant TFEBs. In the presence of glutaraldehyde, TFEB-K274Q/K279Q formed fewer dimers than TFEB-WT and TFEB-K116Q (Figs 5I and EV4K). Taken together, these results suggest that acetylation at K274/K279 by GCN5 hinders the binding of TFEB to DNA by interfering with TFEB dimerization.

Figure 4. Acetylation by GCN5 suppresses TFEB transcriptional activity.
A RT–qPCR analysis of the expression of TFEB target genes in WT, GCN5 KO, and GCN5/TFEB DKO HEK293 cells.
B RT–qPCR analysis of dMit target gene expression in Drosophila larval fat body with silencing of dGcn5.
C Expression of TFEB target genes in HEK293 cells transfected with or without TFEB-WT or TFEB-3KR.
D, E Luciferase activity measured in WT, GCN5 KO and GCN5/TFEB DKO HEK293 cells (D), and in HEK293 cells with TFEB-WT or TFEB-3KR transfection (E). The cells were transfected or co-transfected with a TFEB-luciferase reporter. 3KR: Lys 116, Lys 274, and Lys 279 were replaced by Arg.

Data information: In this Figure, data are presented as mean ± SEM; n = 3 independent experiments; *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t-test.
Figure 5. Acetylation at K274 and K279 disrupts the dimerization and DNA binding of TFEB.

A Subcellular localization of TFEB in WT or GCN5 KO HEK293 cells treated with or without Torin1 (Scale bars, 10 μm).

B Subcellular localization of Flag-tagged TFEB or TFEB mutants in HEK293 cells treated with or without Torin1. Cells were stained with anti-Flag antibody (Scale bars, 10 μm).

C ChIP-qPCR analysis of TFEB binding to the promoter of its target genes CLCN7, GLA, and CTSD. Normal mouse IgG and primers against the upstream region lacking CLEAR sites (up) were used as negative controls (mean ± SEM; n = 3 independent experiments; *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t-test).

D Electrophoresis mobility shift assay of TFEB binding to the promoter of GLA. A DNA fragment from the GLA promoter containing the TFEB-binding site was incubated with purified recombinant TFEB or each of the TFEB mutants, and was subjected to electrophoresis. Asterisk indicates the DNA fragment from the GAPDH promoter which was used as a TFEB non-binding DNA control.

E Molecular dynamics snapshot of homodimer formation by human MITF and MITF mutants. K243 and K248 in the bHLH-Zip domain of human MITF correspond to K274 and K279 in human TFEB, respectively. Steric interference is shown by yellow lines. Images were created using Chimera (http://www.cgl.ucsf.edu/chimera/) and Protein Data Bank (PDB) accession 4ATH.

F Co-precipitation of Flag-tagged TFEB and TFEB mutants with Myc-TFEB in HEK293T cells. Immunoprecipitation was carried out with anti-Myc beads, and the precipitates were analyzed using anti-Flag.

G, H Co-precipitation of Flag-tagged TFEB and TFEB mutants with Myc-MITF (G) or TFE3 (H) in HEK293T cells.

I TFEB homodimer formation detected by glutaraldehyde (GA) cross-linking. Purified recombinant GST-tagged TFEB or TFEB mutants were incubated with or without glutaraldehyde; then, the products were analyzed by immunoblotting using anti-TFEB.

Source data are available online for this figure.
So far, phosphorylation-dependent translocation from the cytoplasm to the nucleus is the main regulatory pathway for TFEB activation [4–6,42]. In order to provide more direct evidence that GCN5 modifies TFEB in the nucleus, thereby affecting its DNA binding, we examined the effect of GCN5 on the dimerization of a TFEB mutant, in which both of the known phosphorylation sites S142 and S211 were substituted by alanine (TFEB-2SA) and were specifically localized in the nucleus (Fig EV4L). We observed a strong co-precipitation between TFEB-2SA-Flag and TFEB-2SA-Myc, which was decreased by overexpression of GCN5 and increased by GCN5 knockdown (Fig EV4M). Accordingly, induction of TFEB-2SA-Flag enhanced the activity of the TFEB-luciferase reporter, and the
Finally, we applied a Drosophila driver, we ectopically expressed the human microtubule-associated protein hTau in Drosophila eyes. This led to the formation of hTau aggregates and neurotoxicity which presented as a rough ocular phenotype [45,46] (Fig 7A, C and D). The introduction of exogenous dMitf improved the degenerative phenotype and reduced hTau aggregation, which were missing in CQ-fed Drosophila (Fig 7A, C and D), supporting previous observations in mouse brain models [47]. Surprisingly, the introduction of dMitf-2KQ aggravated rather than improved the aggregation and neurotoxicity of hTau (Fig 7A, C and D). The phenotype was very similar to that of dMitf KD Drosophila (Fig 7A, C and D), suggesting dMitf-2KQ may play the role of dominant negative mutant. In addition, knocking down dGcn5 in the Drosophila eyes reduced hTau aggregation and mitigated the neurotoxic phenotype (Fig 7B–D). In Drosophila with dAtg7 KD or fed with CQ, the effect of dGcn5 silencing disappeared (Fig 7B–D), which suggests that it is dependent on the activation of autophagy.

**Discussion**

To date, the study of GCN5 in cell metabolism has mainly focused on its regulation of PGC-1α, the transcriptional coactivator for the expression of genes involved in mitochondrial functional. Here, our results demonstrate that GCN5 is a direct modulator of TFEB, the master transcription factor for cell autophagy and lysosomal biogenesis. This mechanism suggests that GCN5 not only regulates the recycling of nutrients and energy in cells, but also plays a role in the digestion and clearance of intracellular protein aggregates and impaired organelles, which are crucial for cell metabolism and growth.

By demonstrating that GCN5 modifies TFEB through acetylation at specific sites, we have uncovered novel molecular mechanisms for the regulation of this important transcription factor. Our discovery will also contribute to the dissection of lysosome-related intracellular functions, especially the coupling of cell metabolism status with lysosome biogenesis. While the regulation of TFEB through its phosphorylation status has been widely recognized, our results clearly show that GCN5-mediated acetylation inhibits TFEB transcriptional activity without affecting its subcellular localizations. The evidence provided by this study strongly suggests that the acetylation by GCN5 serves as an additional step for controlling the activity of TFEB after it enters the nucleus. Despite the lack of direct evidence, it has recently been proposed that TFEB dimerization occurs in the cytoplasm and is necessary for nuclear translocation of TFEB [48]. With co-immunoprecipitation analysis, it has been
shown that the phosphorylation-disabled TFEB-S142A/S211A can form dimers with the phosphorylation-mimic TFEB-142D/211D, and the ratio of phosphorylated-non-phosphorylated TFEB heterodimer to non-phosphorylated TFEB homodimer may affect the efficiency of TFEB nuclear entry [48]. However, it seems that the TFEB dimer may be formed in the nucleus. Theoretically, the different localizations of phosphorylated TFEB and non-phosphorylated TFEB may prevent them from forming dimers. In addition, we show that acetylation of nuclear TFEB can significantly inhibit its dimerization. While the possibility that acetylation may disrupt dimeric TFEB cannot be ruled out, another possibility is that replacing S142 and S211 with aspartic acid may not mimic the phosphorylation of TFEB in the detection of dimer formation, because we observed an obvious distribution of TFEB-S142D/S211D in the cell nucleus (Appendix Fig S1).

K116 has previously been reported as an acetylation-competent residue on TFEB [11,16]; however, the effect of acetylation at this site on TFEB activity was unclear. While deacetylation of K116 by Sirt1 was linked to enhanced lysosomal biogenesis [11], its acetylation in cells treated with deacetylase inhibitors was involved in the activation of lysosomal and autophagy processes [16]. We found that K116 is a target site of GCN5, and its acetylation leads to a
decrease in the expression of TFEB target genes (Appendix Fig S2). However, compared with K274 and K279, which are more conserved between species, acetylation of K116 has little effect on the dimerization and DNA binding of TFEB. Structurally, K116 is located near the transactivation domain of TFEB, which contributes to TFEB transcriptional activity mainly by recruiting transcriptional coactivators and facilitating the assembly of transcription initiation complexes [49,50]. We propose that acetylation at K116 influences the interaction of TFEB with its coactivators, and because K116 is not conserved in other members of the MiT/TFE family, K274 and K279 may play a leading role in regulating TFEB activity. In addition, based on our results from Drosophila showing that overexpression of dMitf-2KQ leads to aggravated aggregation and neurotoxicity of hTau, we propose that acetylation at K274 and K279 may play the role of dominant negative mutation. It is possible that acetylated TFEB competes with deacetylated TFEB for the binding of CARM1 (unpublished data), which is a coactivator of TFEB that regulates the transcription activity of TFEB [51].

It has been shown that mTORC1 exhibits kinase activity in the cell nucleus and acetyltransferase p300 can be directly phosphorylated and activated by mTORC1 [52–54]. In this study, we showed that GCN5 is significantly inactivated in amino acid-starved cells and Torin1-treated cells, which strongly suggests a potential regulatory role of mTORC1 on GCN5. This regulation may coordinate with the mTORC1-controlled nuclear entry–exit events of TFEB [55], enabling the cell to maintain low TFEB transcriptional activity under growth conditions. Upon cell stress, the inactivation of mTORC1 leads not only to accumulation of TFEB in the nucleus, but also to binding of TFEB to the promoters of its target genes through inactivation of GCN5, thus increasing the lysosome biogenesis and autophagy flux. Although the regulatory effect of mTORC1 on GCN5 remains to be confirmed by future studies, our results demonstrate that the GCN5-TFEB pathway plays an important role in clearing protein aggregates in cells, and may be a potential therapeutic target for neurodegenerative diseases.

Materials and Methods

Cell culture and treatment

HEK293, HEK293T, MEF, and HeLa cells were grown in Dulbecco’s modified Eagle Medium (DMEM; Gibco) supplemented with 10% FBS in a 37°C incubator with a humidified, 5% CO2 atmosphere. Drosophila S2 cells were cultured in Schneider’s Drosophila medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FCS in a 25°C incubator.

Unless otherwise stated, the chemicals were used as follows: Torin1, 250 nM, 4 h; MB-3, 50 μM, 16 h; chloroquine, 50 μM, 4 h; bafilomycin A1 (Baf), 100 nM, 4 h; and C646 10 mM, 4 h.

Antibodies

Antibody to dGAPDH is kindly provided by Dr. Wanzhong Ge. Antibodies to Ace-lys (9441), TFEB (4240), Ace-H3 (K9) (9649), Ace-H3 (K27) (4353), histone H3 (4499), 4E-BP1 (9644), and P-4E-BP1 (Thr37/46) (2855) were purchased from Cell Signaling Technology; antibodies to IFT20 (13615), PDLM1 (11674), β-Actin (66009), and p62 (18420) were purchased from Proteintech; antibodies to GFP (M048-3), α-Tubulin (M175-3), Myc (M192-3S), Flag (M185-3B), HA (M180-3S), and GST (M071) were purchased from MBL; antibodies to LC3 (L7543) and Tau (T9450) were purchased from Sigma; antibodies to CTSD (136282), LAMP1 (17768), and GCN5 (365321) were purchased from Santa Cruz; antibody to EGFR (db1025) was purchased from Diagbio.

Drosophila stocks

All Drosophila stocks were reared on a standard cornmeal medium in 25°C incubators under a 12-h:12-h light:dark cycle [56]. CQ (Sigma-Aldrich) as a 100-mM stock solution was freshly prepared in H2O and dissolved into Drosophila media at 10 mM. The GMR-Ga4 Drosophila strain was kindly provided by Dr. Wanzhong Ge. The eg-Ga4 and mCherry-Atg8a Drosophila strains were kindly provided by Dr. Chao Tong. dGcn5 KD (line BL9332) and hTau (line BL51363) were purchased from the Bloomington Stock Center (Bloomington, IN). dMitf KD (line THU3522) and dATG7 KD (line THU1583) were purchased from the Tsinghua Fly Center (THFC). All genotypes of fly strains generated in the paper were added as Appendix Table S1. RNAi efficiency in Drosophila is presented in Appendix Fig S3.

Site-directed mutagenesis and Drosophila transformation

To generate transgenic Drosophila, cDNA encoding full length dGcn5 and dMitf were cloned into pUAST-attB vector. cDNA was reversely transcribed using M-MLV reverse transcription reagents (Promega) with RNA extracted from Drosophila head. pUAST-attB vector containing dMitf-Flag was used as the template to generate various dMitf mutants via site-directed mutagenesis. pUAST-attB vector containing Flag-tagged dMitf, dMitf-2KQ, and dGcn5 were sequence confirmed and germline transformed into Drosophila (Core Facility of Drosophila Resource and Technology, SIBCB, CAS). All of the genetic manipulations in our study were based on Drosophila w1118.

Transfection

Transient transfection of DNA in mammalian cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were analyzed 18–24 h after transfection. Transient transfection of DNA in Drosophila S2 cells was performed using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instructions. S2 cells were analyzed 48 h after transfection.

For RNA interference in mammalian cells, siRNA duplexes designed against conserved targeting sequences were transfected using Lipofectamine 2000 for 72 h. The following siRNA duplexes were used: GCN5 siRNA: CCGGAAGCCUCAGGCAUG; TFEB siRNA: AAACGGAGCCUACUGAACA. RNA interference in Drosophila was described in [Ref. 57]. First, dsRNA targeting dGcn5 is generated by a two-step PCR and in vitro transcription. In the first step, T7 RNA polymerase promoter sequence was fused to the sequences of dGcn5. These are linked to T7 sequences to enable an in vitro transcription with T7-RNA polymerase. 1 μg PCR product was used as a template. 100 μg double-stranded RNAs (dsRNAs) were generated in a 50 μl transcription reaction using the RiboMAX
Large Scale RNA Production Systems (Promega). The primers used were as follows: dGen5 F: 5’-CTCCTATAGGGAAGCGGAATGGGC CGACCGACG-3’; dGen5 R: 5’-CTCCTATAGGGAAGCGGAATGGGC CGACCGACG-3’. T7 promoter primer: GAATTAATACGACTCAG TATTAGGAGA. S2 cells were resuspended in serum-free medium and incubated with the dsRNAs at room temperature for 30 min. Cells were analyzed 72 h after transfection.

**RNA extraction and quantitative PCR**

Total RNA was extracted from cells or Drosophila tissues using TRIzol (Invitrogen). cDNA was reversely transcribed using M-MLV reverse transcription reagents (Promega). Quantitative PCR was performed with SYBR Premix Ex Taq (TaKaRa) on a 7300 Real-Time PCR System (Applied Biosystems). Gene expression levels were calculated according to the 2−ΔΔCT method and normalized against β-Actin or Act5C (Drosophila). Primers used for RT-PCR were listed in Appendix Table S2 (human cells) and Appendix Table S3 (Drosophila).

**Immunostaining**

Cells were cultured on coverslips and fixed in 4% formaldehyde for 10 min at room temperature. After washed three times with PBS, cells were incubated in PBS containing 10% FBS to block non-specific sites of antibody adsorption. Then, the cells were incubated with primary and secondary antibodies in PBS containing 0.1% saponin and 10% FBS. Drosophila fat bodies were dissected in cold PBS from the third-instar larvae and then fixed in 4% formaldehyde for 20 min. After washed three times with PBST (PBS + 0.1% Triton X-100), tissues were incubated with DAPI at room temperature for 1 h. After extensive wash, samples were mounted in vector shield.

Confocal images were captured in multitracking mode on an LSM800 Meta laser-scanning confocal microscope (Carl Zeiss) with a 63× Plan Apochromat 1.4 NA objective and analyzed with the ZEN 2012 software.

**Light microscopy and qualitative analysis of eye phenotypes**

The Drosophila eye images were captured using a Nikon SMZ18 microscope. Ten images with sequential focal points were taken to cover an entire eye, and these images were reconstituted to a fully focused final image using the NIS-Elements (version 3.0).

To apply quantitative analysis, we randomly selected 30 3-day-old female flies per genotype and classified phenotypic severity according to the severity categories described previously (Yusha Wang et al.); and category 3 had black spots of necrotic tissue on their eyes (severe).

**Stable cell line construction**

pEP-GCN5-KO plasmid was made by cloning the target DNA sequence of human GCN5 (GGGGGGATCCGGCTCGACC) into a pEP-KO Z1779 vector using Sapl. GCN5 KO HEK293/HeLa cells were created by transient transfection of pEP-GCN5-KO plasmid followed by selection with 2.5 μg/ml puromycin. pEP-TFEB-KO plasmid was made by cloning the target DNA sequence of human TFEB (AGTACCTGTCGGAGACCTAT) into a pEP-KO Z1779 vector using Sapl. GCN5/TFEB DKO HEK293/HeLa cells were created by transfection of pEP-TFEB-KO plasmid into GCN5 KO HEK293/HeLa cells followed by selection with 2.5 μg/ml puromycin.

HEK293 cells stably expressing GFP-p62 were created by transient transfection followed by selection with G418 (500 μg/ml) for 2 weeks.

**Immunoblotting and immunoprecipitation**

Mammalian cells were harvested and lysed in Nonidet P-40 (NP-40) lysis buffer (20 mM Tris–HCl, pH 7.5, 0.5% NP-40, 1 mM MgCl2, 137 mM NaCl, 1 mM CaCl2, 10% glycerol, 1 mM Na3VO4, 10 mM NaF, 1 mM TFA, 5 mM NAM). Drosophila cells were harvested and lysed in lysis buffer (25 mM Tris–HCl, pH 8.0, 27.5 mM NaCl, 20 mM KCl, 25 mM sucrose, 10 mM EDTA, 10 mM EGTA, 0.5% NP-40, 10% glycerol, 1 mM DTT). All buffers were used throughout processing contained protease inhibitors. Proteins were denatured and resolved on sodium dodecyl sulfate-polyacrylamide gels (SDS–PAGE) and then transferred to a polyvinylidene difluoride membrane. After blocking with 5% (w/v) bovine serum albumin, the membrane was stained with the corresponding primary antibodies and secondary antibodies. Specific bands were analyzed using an Odyssey infrared imaging system (LI-COR Biosciences). Protein bands were quantified using the ImageJ software.

For immunoprecipitation, cell lysates were mixed with antibodies at 4°C overnight, followed by the addition of protein A/G agarose beads for 2 h. Then, immunocomplexes were washed five times using lysis buffer and subjected to immunoblotting.

Nonionic detergent soluble and insoluble hTau fractions were prepared using a previously described method [59]. Briefly, Drosophila heads were homogenized in TNE buffer (10 mM Tris–HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA) containing protease inhibitor cocktail and 0.5% NP-40. After centrifugation at 10,000 g for 10 min at 4°C, supernatant was collected as soluble fraction. The pellet was washed once in TNE buffer and solubilized in TNE buffer containing 1% SDS. Protein concentrations in the supernatants were measured with a BCA protein assay kit. Equivalent amounts of soluble and insoluble proteins from different flies were separated by SDS–PAGE for immunoblotting.

**Glutaraldehyde cross-linking**

In a 20 μl reaction system, 100 μg purified proteins of GST-tagged TFEB or TFEB mutants were incubated with 0.003% of glutaraldehyde within reaction buffer (10 mM Tris pH 8.0, 140 mM NaCl, 0.5% NP-40) at 25°C for 5 min. The reaction was stopped by adding protein sample buffer, and the samples were analyzed by immunoblotting.

**Subcellular fractionation**

Cell pellets were washed with PBS and resuspended in lysis buffer (10 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT). The suspension was put on ice for 30 min. After centrifugation at
500 g for 5 min at 4°C, the supernatant was collected as cytosolic fraction. The pellet was washed twice with lysis buffer and resuspended in 0.5% NP-40 lysis buffer. After centrifugation at 15,000 g for 15 min, the resultant supernatant was used as the nuclear fraction. All buffers used throughout processing contained protease inhibitors.

**ß-Hexosaminidase assay**

Lysosomal ß-hexosaminidase assay was described in [Ref. 60]. Equal numbers (1 × 10⁶ cells) of HEK293 cells (WT or GCN5 KO) were lysed in 150 μl 0.1% Triton X-100 containing protease inhibitor cocktail for 20 min and centrifuged at 10,000 g. 20 μl of 1 mM p-nitrophenyl-N-acetyl-14-d-glucosaminide (p-NAG) (Sigma-Aldrich, Cat #: N9376) was incubated with 20 μl of each clarified sample at 37° C for 1 h. 250 μl of 0.1 M Na₂CO₃/NaHCO₃ solution was added to stop the reaction. The reaction product was measured by reading the absorbance at 405 nm immediately.

**LysoTracker staining**

Cells were cultured on coverslips and incubated for 30 min with 50 nM LysoTracker red (DND-99; Life Technologies, L-7528). Cells then were washed twice with PBS and visualized in PBS containing DAPI.

*Drosophila* fat bodies were dissected in cold PBS from the third-instar larvae and incubated in PBS containing 1 μM LysoTracker red at room temperature for 45 min. After washed three times with PBS, tissues were transferred to Schneider’s *Drosophila* medium (Sigma-Aldrich) with DAPI on glass slides, covered, and immediately photographed live on LSM800 Meta laser-scanning confocal microscope. Acidic vesicles were counted manually using ImageJ software analysis.

**FACS analysis**

Cells were cultured in 50 nM LysoTracker red for 30 min. Then, cells were trypsinized and washed with PBS. Red lysosomal fluorescence of 10,000 cells per sample was determined by flow cytometry using the Cytomic FC 500MCL (BECKMAN COULTER).

**Recombinant protein purification and in vitro acetylation assay**

GST-TFEB was expressed in *Escherichia coli* BL21. Bacteria were treated with 0.1 mM IPTG at 30°C to induce protein expression, and were harvested and resuspended in lysis buffer (PBS containing 0.5% Triton X-100, 2 mM EDTA, and 1 mM PMSF), followed by ultrasonication. The recombinant TFEB proteins were purified using glutathione-sepharose 4B beads. Then, the beads were centrifuged by gentle rotation at 4°C and washed three times with 10 ml lysis buffer, then suspended with TEV cleavage buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40, 1 mM DTT, and 1 mM EDTA). 80 μl TEV protease (kindly provided by Sun’ lab) was added, and incubation was performed overnight by gentle rotation at 4°C. The supernatant (containing recombinant TFEB proteins) was obtained by centrifuging.

Myc-GCN5 protein was purified from HEK293T cells 24 h after transfection by immunoprecipitation with anti-Myc affinity beads (Selleck). For in vitro acetylation assay, TFEB protein (10 mg) was incubated with Myc-GCN5 immunoprecipitated from cell lysate, in the presence of acetyl-coenzyme A (4 mg) and 10 μl 5× HAT assay buffer (250 mM Tris–HCl, pH 8.0, 5 mM diithiothreitol, 50% glycerol, 0.5 mM EDTA) in a total volume of 50 μl. The contents were gently mixed and placed in a 30°C shaking incubator for 1 h. Then, protein loading buffer was added to the reaction and boiled for 5 min. The reaction products were separated by SDS–PAGE for immunoblotting or Coomassie Blue staining. Specific band was cut off and subjected to HPLC-MS/MS.

**Fluorometric GCN5 activity assay**

Myc-GCN5 was immunoprecipitated from HEK293T cells. The immunoprecipitated proteins were incubated with acetyl-CoA and peptide substrate histone H3 at 37°C for 15 min according to the manufacturer’s protocol (Active Motif #56100). The reaction was stopped with Stop Solution, followed by further incubation with Developer Solution for 15 min in the dark at room temperature. The activity of GCN5 was assessed by measuring the fluorescent emission at 460 nm following excitation at 380 nm.

**HPLC-MS/MS**

To identify the acetylation site of TFEB by mass spectrometry, the gel band of acetylated TFEB was cut off. In-gel digestion of TFEB was performed with MS-grade modified trypsin (Promega) at 37°C overnight. The digested peptides were loaded on an in-house packed capillary reverse-phase C18 column (15 cm in length, 3 mm particle size, 100 mm ID 3 360 mm OD, 100 Å pore diameter) connected to an Easy LC 1000 system. The samples were analyzed with a 180 min-HPLC gradient from 0% to 100% buffer B (0.1% formic acid in acetonitrile) at 300 nl/min. The eluted peptides were ionized and directly introduced into a Q-Exactive or Fusion mass spectrometer (Thermo) using a nano-spray source. Survey full-scan MS spectra (m/z 300–1,800) were acquired in the Orbitrap analyzer with resolution r = 70,000 at m/z 400.

**Luciferase assays**

The TFEB-luciferase construct which contains 4× CLEAR sites before luciferase reporter was provided by Dr. A. Ballabio [3]. HEK293 cells were transfected with indicated Flag-tagged TFEB or mutant plasmids for 24 h using Lipofectamine 2000 (Invitrogen). Then, cells were transfected with TFEB-luciferase construct for another 48 h, and luciferase assays were performed using a Dual-Luciferase Reporter Assay System (Promega) based on the protocol provided by the manufacturer.

**ChIP assay**

HeLa cells were transfected with Flag-tagged TFEB or mutants for 48 h. Then, cells were incubated in 1% formaldehyde for 10 min at room temperature to crosslink DNA to associated proteins. A final concentration of 0.125 M glycine was added to stop the cross-linking reaction. Cells were washed twice by cold 1× PBS and lysed with SDS lysis buffer (50 mM Tris–HCl, pH 8.1, 10 mM EDTA, pH 8.0, 1% SDS) containing protease inhibitors. After 30 min of
incubation on ice, lysates were centrifuged for 5 min at 800 g and 4°C, and the nuclear pellets were collected and sonicated in SDS lysis buffer. For immunoprecipitation, 200 μl of chromatin was diluted 1:5 in ChIP dilution buffer (16.7 mM Tris–HCl, pH 8.1, 0.01% SDS, 1.1% Triton X-100, 2 mM EDTA, 167 mM NaCl) and 1% of the diluted sample was set aside for input. The diluted sample was precleared with Protein G beads (Bioworld) at 4°C for 1 h. The precleared lysates were incubated overnight with 1 μg of anti-Flag M2 affinity gel (SIGMA). After immunoprecipitation, the beads were washed at room temperature (for 5 min each) with 700 μl of low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 150 mM NaCl), high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholic acid, 1 mM EDTA, 20 mM Tris, pH 8.1), and TE buffer (10 mM Tris–HCl, 1 mM EDTA at pH 8.0). Elution was performed twice in 250 μl of fresh elution buffer (1% SDS and 0.1 M NaHCO3) for 15 min at room temperature. 20 μl of 5 M NaCl was added, and samples were incubated overnight at 65°C to reverse the crosslinks. Input DNA was diluted in freshly made elution buffer to a volume of 500 μl, and crosslink reversal was performed. After crosslink reversal, samples were digested with 20 μg of proteinase K for 2 h at 45°C, and DNA was recovered by standard methods in 20 μl of 10 mM Tris–HCl at pH 8.0. 1 μl of DNA was used for each quantitative PCR. The quantitative PCR data were analyzed as described previously [61]. The primers used for ChIP-qPCR were listed in Appendix Table S4.

**EGFR degradation assay**

After cultured in serum-free DMEM for 12 h, HEK293 or GCN5 KO HEK293 cells incubated on ice in serum-free DMEM medium containing 200 ng/ml of EGF for 15 min. Next, the cells were washed with PBS and cultured in serum-free DMEM at 37°C. At determined time points, the cells were lysed and subjected to immunoblot with EGFR antibody.

**Electrophoretic mobility shift assay**

Recombinant protein TFEB and TFEB mutants were purified from *Escherichia coli*. GLA promoter DNA fragments and non-specific DNA (GAPDH promoter region) were amplified using primers listed in ChIP assay column. DNA was purified by QiAquick PCR purification kit and eluted with ultrapure water. 1 μg of DNA fragments was incubated with purified proteins (25–50 μg) in the *in vitro* binding buffer (10 mM Tris–HCl, pH 7.5, 50 mM KCl, 5 mM MgCl2, 1 mM DTT, 2.5% glycerol, 0.05% NP-40, 5 μg/ml salmon sperm DNA) at room temperature for 1 h. Samples were separated by the 1.5% agarose gel and stained with ethidium bromide. The shift of the bound DNAs was visualized under ultraviolet (UV) light.

**Expanded View** for this article is available online.

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**Author contributions**

WL, YW, and ZG designed the experiments. YW, YH, jl, ZY, JZ, and MX performed the experiments. CP performed the mass spectrometry. WL and YW wrote the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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