Cell Metabolism

Transcription Factor EB Controls Metabolic Flexibility during Exercise

Graphical Abstract

Highlights

- TFEB regulates mitochondrial biogenesis and function in muscle
- Glucose homeostasis in skeletal muscle requires TFEB
- The effects of TFEB on muscle metabolism are independent from PGC1α
- TFEB coordinates metabolic flexibility during physical exercise

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In Brief

Mansueto et al. show that TFEB acts as a central coordinator of skeletal muscle insulin sensitivity, glucose homeostasis, lipid oxidation, and mitochondrial function in the adaptive metabolic response to physical exercise in a PGC1α-independent manner.
Transcription Factor EB Controls Metabolic Flexibility during Exercise

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SUMMARY

The transcription factor EB (TFEB) is an essential component of lysosomal biogenesis and autophagy for the adaptive response to food deprivation. To address the physiological function of TFEB in skeletal muscle, we have used muscle-specific gain- and loss-of-function approaches. Here, we show that TFEB controls metabolic flexibility in muscle during exercise and that this action is independent of peroxisome proliferator-activated receptor-γ co-activator1α (PGC1α). Indeed, TFEB translocates into the myonuclei during physical activity and regulates glucose uptake and glycogen content by controlling expression of glucose transporters, glycolytic enzymes, and pathways related to glucose homeostasis. In addition, TFEB induces the expression of genes involved in mitochondrial biogenesis, fatty acid oxidation, and oxidative phosphorylation. This coordinated action optimizes mitochondrial substrate utilization, thus enhancing ATP production and exercise capacity. These findings identify TFEB as a critical mediator of the beneficial effects of exercise on metabolism.

INTRODUCTION

Exercise elicits several beneficial effects by acting on mitochondrial content/function, fatty acid oxidation, and glucose homeostasis (Hawley, 2002; Holloszy and Coyle, 1984; Holloszy et al., 1996). Indeed, muscle activity is important to counteract disease progression in diabetes, obesity, and metabolic syndrome. The signaling pathways that control the contraction-mediated beneficial effects on mitochondria and glucose/lipid homeostasis are distinct from insulin signaling and mainly rely on AMPK and PGC1α. We have recently found that exercise leads to nuclear translocation of the helix-loop-helix leucine zipper transcription factor EB (TFEB) (Medina et al., 2015), an important regulator of lysosomal biogenesis and autophagy (Sardiello et al., 2009; Settembre et al., 2011). Upregulation of TFEB has been found in several tissues after food deprivation, including liver and skeletal muscle. We have previously shown that in liver, TFEB regulates genes involved in lipid catabolism, fatty acid oxidation, and ketogenesis (Settembre et al., 2013). Some of these effects are elicited by TFEB-mediated induction of PGC1α (Settembre et al., 2013), a transcriptional coactivator, which interacts with and enhances the activity of transcription factors involved in mitochondrial biogenesis, glucose homeostasis, and lipid oxidation (Kelly and Scarpulla, 2004; Puigserver et al., 1998).

In the presence of nutrients, TFEB is sequestered in the cytoplasm by mTORC1-mediated phosphorylation, whereas in nutrient-depleted conditions, mTORC1 is inactive and dephosphorylated TFEB translocates to the nucleus, where it induces the transcription of target genes (Martina et al., 2012; Rocznia-Ferguson et al., 2012; Settembre et al., 2012). The dephosphorylation of TFEB is mediated by the calcium-dependent phosphatase, calcineurin, which is necessary for TFEB activation (Medina et al., 2015). Importantly, exercise-dependent calcium influx activates calcineurin, which dephosphorylates TFEB, leading to nuclear localization. The calcineurin-mediated induction of TFEB is independent from mTORC1 activity, indicating that calcium-dependent signaling is a rate-limiting step of TFEB activation (Medina et al., 2015).

Previous studies implicated calcineurin in a variety of physiological processes, in particular in skeletal muscle adaptation...
Figure 1. TFEB Induces Mitochondrial Biogenesis

(A) Genes involved in lipid and glucose metabolism that were affected by TFEB expression are shown in colored circles. The upregulated (red circles) and downregulated genes (green circles) are shown in TFEB-overexpressing and TFEB KO, respectively. The genes were divided into functional sub-categories.

(B–D) EM analysis of skeletal muscles from WT mice transfected with AAV2.1-GFP (B) and AAV2.1-TFEB (C), and from TFEB KO mice (D). Normal mitochondria are indicated by black arrows (B and C), while abnormal mitochondria in TFEB KO muscle (D) are indicated by empty arrows. Accumulation of glycogen is shown by white arrows in (C). The scale bars represent 500 nm.

(E and F) Morphometrical analyses of mitochondrial number (E) and size (F) in TFEB-overexpressing and TFEB KO muscles compared to controls. Error bars represent mean ± SE, n = 3; **p < 0.01.

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to exercise (Gehlert et al., 2015). Muscle-specific transgenic mice that overexpress an activated form of calcineurin show increased glucose uptake, glycogen accumulation, and lipid oxidation (Long et al., 2007). Interestingly, calcineurin promotes the nuclear translocation of another family of transcription factors, NFAT, which, depending on the type of physical activity, modulate the expression of the different myosin isoforms (Calabria et al., 2009; Long et al., 2007; McCullagh et al., 2004).

Here we show that the calcineurin-TFEB axis plays a major role in the metabolic adaptations that occur during physical exercise. By using gain- and loss-of-function approaches, we show that TFEB regulates mitochondrial biogenesis and glucose uptake independently of PGC1α. Indeed, TFEB controls genes involved in glucose metabolism such as GLUT1 and GLUT4, hexokinase I and II, TBC1 domain family member 1 (TBC1D1), and glycogen synthase (GYS), leading to glycogen accumulation to sustain energy production during exercise.

RESULTS

Genome-wide Analyses Identified Glucose-Related and Mitochondrial Genes as Downstream Targets of TFEB

We previously demonstrated that TFEB promotes lipid catabolism in the liver and protects against diet-induced weight gain and insulin resistance (Settembre et al., 2013). Here we studied the physiological relevance of TFEB in skeletal muscle, an important insulin- and autophagy-dependent tissue (Grumati and Bonaldo, 2012; Mammucari et al., 2007; Masiero et al., 2009). Transcriptome analysis was performed by whole-genome gene expression profiling experiments (SuperSeries-GSE62980) in skeletal muscle from both TFEB-overexpressing and TFEB knockout (KO) mice. Overexpression of Tcfeb, the murine homolog of human TFEB, in muscle was achieved by means of intramuscular viral-mediated gene transfer using the adeno-associated virus (AAV) system. Adult mice were injected intramuscularly with either AAV2.1-CMV-TFEB or AAV2.1-CMV-GFP control vector and animals were sacrificed after 21 days, a time that allows efficient TFEB expression (Figure S1A, available online). Muscle-specific conditional TFEB KO mice were generated by crossing Tcfeb floxed (Settembre et al., 2013) with MLC1f-Cre transgenic mice (Bothe et al., 2000). Efficiency and specificity of the gene deletion were confirmed by quantitative real-time PCR analysis on multiple tissues (Figure S1B).

Overexpression of TFEB in muscle resulted in the upregulation of 1,514 genes and the downregulation of 1,109 genes (GSE62975), while genetic ablation of TFEB increased 496 genes and suppressed 458 genes (GSE62976). The up- or downregulated genes are highlighted in red and green, respectively, in Tables S1 and S2. To identify the main cellular compartments (CCs) and the principal biological process (BPs) for which the TFEB-dependent genes were enriched, we performed a gene ontology enrichment analysis (GOEA). The GOEA was performed on the lists of genes whose expression was either increased or decreased in transfected muscle or in the TFEB KO mice. Interestingly, several gene categories related to cellular metabolism, including lipid and glucose homeostasis, were found upregulated in TFEB-overexpressing muscle and downregulated in TFEB KO (Figure 1A; Table S3). Strikingly, genes involved in mitochondrial biogenesis were oppositely regulated by gain- and loss-of-function approaches. Indeed, 38 genes involved in mitochondrial function were induced in AAV2.1-TFEB-infected muscles (Table S4), while 73 genes were inhibited in TFEB KO muscles (Table S5).

To better identify the network of genes regulated by TFEB in muscle, we performed sequence analysis to identify putative TFEB target sites, previously referred to as CLEAR sites (coordinated lysosomal expression and regulation) (Palmieri et al., 2011), in the promoter regions of the downregulated genes in TFEB KO mice. Interestingly, we found that 79% of these genes contain a CLEAR sequence and are, therefore, potential direct targets of TFEB (Table S6).

TFEB Regulates Mitochondrial Biogenesis in Muscle

To examine potential effects of TFEB in mitochondrial function, we analyzed mitochondrial morphology in muscles overexpressing or lacking TFEB. Electron microscopy (EM) analyses showed a striking increase of mitochondrial density and volume in TFEB-overexpressing muscles (Figures 1B, 1C, 1E, and 1F). Interestingly, mitochondrial density and size were normal in the TFEB KO muscles (Figures 1E and 1F). Consistent with the EM data, increase of mitochondrial DNA (mtDNA) was found in TFEB transgenic muscles, while no differences were observed in TFEB KO muscles (Figure 1G). However, while the cristae shape, matrix density, and outer membrane morphology were normal in TFEB-overexpressing muscles (Figure 1C), abnormalities were found in approximately 10% of the mitochondria from TFEB KO muscles (Figures 1D and 1H). An increase in the number of mitochondria was also observed in C2C12 muscle cells transfected with TFE3-GFP, as detected by immunofluorescence confocal and confirmed by EM analyses (Figures S2A and S2B). Quantitative real-time PCR also revealed an increase of mtDNA content in TFEB-overexpressing cells (Figure S2C).

Importantly, quantitative real-time PCR analysis revealed that TFEB overexpression in muscle and in C2C12 cells induces the expression of many genes involved in mitochondrial biogenesis and function, including the master gene of mitochondrial biogenesis, PGC1α, a known direct target of TFEB (Settembre et al., 2013) (Figures 2A and 2D). Moreover, another PGC-1 family member, PGC1β, was also upregulated by TFEB overexpression. Consistently, we found a significant induction of peroxisome proliferator-activated receptor α (PPARα), PPARβ/δ, and PPARγ in TFEB-overexpressing muscles. However, TFEB deletion did not affect the expression of PGC1α/β and PPAR genes, with the exception of PPARβ, which was downregulated. In order to elucidate the possible mechanisms underlying the induction of (G) mtDNA analysis of muscles infected with AAV2.1-TFEB and from TFEB KO compared to WT mice. Quantitative real-time PCR of mtDNA copy numbers. Data are shown as mean ± SE, n = 3; *p < 0.01. (H) Quantification of abnormal mitochondria in TFEB KO gastrocnemius (GCN) muscles compared to WT muscles. Data are expressed as percentage of abnormal mitochondria on total mitochondria of 16 pictures (for detailed description of the criteria, see Experimental Procedures). Error bars represent mean ± SE; *p < 0.05.
A Transcriptional Regulation of Mitochondrial Biogenesis

B Transcriptional Control of Mitochondrial Bioenergetic Function

C

D

E

Citrate Synthase Complex I Complex II Complex III Complex IV

F

G

H

TMRM signal (% of initial value)

I

Protein carbonylation
mitochondrial biogenesis observed in TFEB-transfected muscles, we examined the expression of nuclear respiratory factors 1 and 2 (NRF1 and NRF2). The mRNA levels of NRF2 were increased, as well as NRF downstream genes, including mitochondrial transcription factor A (TFAM). Chromatin immunoprecipitation (ChIP) experiments confirmed the direct recruitment of TFEB on NRF-1 and NRF-2 promoters (Figure 2B), but not on TFAM promoter (data not shown). Importantly, overexpression of TFEB in skeletal muscle increased the expression of mitochondrial enzymes. Subunits of the four respiratory chain complexes and the ATP synthase, as well as genes encoding electron transport and tricarboxylic acid cycle proteins, were induced by TFEB overexpression and were reduced by TFEB deletion (Figure 2A).

TFEB deletion did not alter NDUFA9 and COX5a expression but significantly reduced the level of SDHA protein (Figures 2C and 2D). To better characterize the involvement of TFEB in mitochondrial respiration, we analyzed the specific activities of enzymes involved in oxidative phosphorylation. Biochemical analysis of muscle samples infected with AAV2.1-TFEB compared to wild-type (WT) muscles showed increase of citrate synthase, mitochondrial respiratory chain complex I (CI), CI, CIII, and CIV activities (Figure 2E). Consistent with the western blot analyses, TFEB deletion led to decrease of CI activity, the complex that contains the SDHA flavoprotein, while the other respiratory complexes had normal activities (Figure 2E). The changes in respiratory chain activities were corroborated by histochemical analyses for COX and SDH activity in AAV2.1-TFEB-transfected and TFEB KO muscles. Indeed, only SDH activity was greatly reduced in the absence of TFEB, while both COX and SDH were increased in TFEB-overexpressing muscles (Figure 2F).

To further investigate the role of TFEB in mitochondrial function, we generated an inducible muscle-specific transgenic mouse line. Acute activation of TFEB by tamoxifen treatment in adult mice recapitulated the phenotype of AAV2.1-TFEB overexpressing and WT mice. The CLEAR elements of NRF1 and NRF2 promoters are depicted as red boxes. TSS (transcriptional start site) indicates the first codon. The histograms show the amount of immunoprecipitated DNA as detected by quantitative real-time PCR assay. Data represent mean ± SE of three independent experiments; **p < 0.01.

TFEB Regulates Mitochondrial Biogenesis in Skeletal Muscle through a PGC1α- and PGC1β-Independent Mechanism

Both PGC1α and PGC1β are master regulators of mitochondrial biogenesis and oxidative metabolism. However, recent findings suggest the presence of an independent pathway that regulates mitochondrial biogenesis during exercise (Rowe et al., 2012). Therefore, we examined whether TFEB is the missing sensor of physical activity that coordinates the metabolic responses independently of PGC1α. First, we checked expression and localization of endogenous TFEB in PGC1α KO mice before and after exercise. TFEB was expressed at lower levels and more cytosolic in PGC1α KO mice when compared to controls (Figures 3A and 3B).
3B). Importantly, exercise restored a normal TFEB expression, induced TFEB nuclear translocation, and triggered upregulation of genes related to mitochondrial biogenesis (Figures 3C and S4A).

EM analysis of overexpressed TFEB in PGC1α KO mice revealed increased mitochondrial volume and density (Figure S4B). TFEB overexpression in PGC1α KO muscle also resulted in increased COX and SDH activity along with increased activities of Cl, II, III, and IV (Figures S4C and S4D). The levels of PGC1α targets such as TFAM, NRF1, and NRF2 were also significantly upregulated by TFEB overexpression, even in the absence of PGC1α (Figure S5A). Importantly, systemic delivery of TFEB in muscle-specific PGC1α KO mice improved their exercise tolerance (Figure 3D). Indeed, TFEB expression was able to restore normal fatigue index when expressed in PGC1α KO muscle (Figure 3E). Altogether, these findings suggest that the induction of mitochondrial biogenesis in TFEB-overexpressing muscles does not depend on the presence of PGC1α.

To determine whether PGC1β may compensate for the lack of PGC1α, we measured the effect of TFEB overexpression on mitochondrial biogenesis in cells that were silenced for PGC1α and PGC1β. Importantly, inhibition of both PGC1 factors did not prevent or reduce TFEB-mediated induction of genes related to mitochondrial biogenesis and mitochondrial respiratory chain activity (Figure S5B).

**TFEB Controls Energy Balance in Skeletal Muscle during Exercise**

Physical activity has a major impact on glucose homeostasis and mitochondrial biogenesis and function. Therefore, we checked whether acute exhausting versus mild and chronic exercise regimens are equally able to activate TFEB. As a readout of TFEB activation, we monitored its nuclear localization. While acute exhausting contraction led to nuclear translocation of TFEB (Figure 4A), mild exercise did not (Figure 4B). However, 7 weeks of training with progressive increase of intensity without reaching exhaustion induced a massive TFEB nuclear translocation with concomitant cytosolic depletion (Figure 4B). Therefore, intensity and duration of training are critical factors that affect TFEB nuclear translocation and transcriptional regulation of genes related to mitochondrial biogenesis and function (Figure 4C).

To determine the physiological consequences of TFEB translocation during physical activity, we examined exercise performance in both TFEB KO and inducible muscle-specific TFEB transgenic mice. High-intensity exercise revealed significant training intolerance of TFEB KO mice compared to controls (Figure 5A). Conversely, acute muscle-specific activation of TFEB enhanced physical performance (Figure 5A). To better understand the exercise intolerance of the TFEB KO mice, we examined energy expenditure during treadmill running. While WT mice maintained constant levels of energy expenditure during physical activity, the TFEB KO mice displayed a drop after 15 min of physical exercise (Figure 5B). Metabolic analyses revealed that in basal condition, TFEB KO mice have a higher respiratory exchange rate (RER) than controls (Figure 5C). These data suggest that TFEB KO mice depend on glucose oxidation more than controls. In addition, while WT mice maintained a relatively constant RER during running period, TFEB KO mice showed a drop in RER after 20 min (Figure 5C). This decrease indicates a shift in substrate usage from glucose to fat metabolism. Finally, we measured glucose and fatty acid levels in muscle and blood from TFEB KO and TFEB transgenic mice before and after exercise. TFEB KO mice showed lower blood glucose levels compared to WT mice in basal condition (Figure 5D). Exercise caused a 50% reduction of blood glucose in TFEB KO, TFEB transgenic, and control mice (Figure 5D). Insulin levels mirrored the changes of blood glucose, as they were reduced in basal condition in TFEB KO mice and dropped after exercise in the different genotypes (Figure 5E).

TFEB KO mice are hypoglycemic, contain dysfunctional mitochondria, and produce less ATP. Thus, we reasoned that they use anaerobic glycolysis to produce energy. Consistent with this hypothesis, we found higher levels of lactate in the blood of TFEB KO mice before and after exercise compared to controls (Figure 5F). Conversely, lactate of transgenic mice was already lower than controls in resting condition and did not increase after exercise (Figure 5F). Therefore, TFEB transgenic mice better utilize glucose for energy production. To further confirm this finding, we measured glycogen levels in muscle. Glycogen levels were remarkably lower in TFEB KO mice and higher in TFEB transgenic in basal condition compared to WT. Enzymatic quantification showed that glycogen content was 3-fold less after TFEB ablation (Figure 5G), while it was 10-fold higher after TFEB overexpression compared to controls (Figure 5G). This was confirmed by periodic acid-Schiff (PAS) staining (Figure 5H).

Exercise led to glycogen consumption in both TFEB KO muscles and controls (Figure 5G). The lower glycogen content detected in sedentary TFEB KO mice explains the decrease of RER...
Figure 4. Exercise Induces TFEB Nuclear Translocation

(A) GCN tissue sections of TFEB KO and WT mice were immunostained with anti-TFEB antibody and counterstained with hematoxylin as indicated. Arrows indicate nuclear localization of endogenous TFEB in WT mice after an acute bout of exercise. The scale bars represent 50 μm.

(B) Endogenous TFEB was immunostained in muscle cryosections of sedentary, 4 days mild exercised, and 7 weeks intense trained WT mice. Arrows indicate TFEB nuclear localization.

(C) Expression analysis of genes related to mitochondrial biogenesis and mitochondrial respiratory chain in WT skeletal muscle before and after acute exercise. Data are shown as mean ± SE, n = 3; *p < 0.05, **p < 0.01.
**A** Distance Run until Exhaustion (m)

**B** Energy Expenditure

**C** Respiratory Exchange Ratio

**D** Glucose (mg/dL) (serum)

**E** Insulin (ng/ml) (serum)

**F** Lactate (mmol/L) (serum)

**G** Glycogen (µg/mg) (muscle)

**H** PAS (PAS-diastase)

**I** FFA (mEq/L) (serum)

**J** FFA (mEq/L) (muscle)

**K** B-Hydroxybutyrate (mM/mg) (muscle)

**L** Glucose Infusion Rate During EU-Clamp

**M** Glucose Uptake Rate During EU-Clamp

**N** Glycogen Synthesis During EU-Clamp

**O** Glucose Uptake in EPI During EU-Clamp

**P** Liver Glucose Output During EU-Clamp

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observed after a 15 min exercise (Figure 5C). Since glycogen is rapidly depleted in TFEB KO mice, the additional need for energy during exhausting exercise requires a switch from glycolysis to fatty acid oxidation. Consistently, while blood free fatty acid concentrations were reduced after exercise in both TFEB KO mice and controls (Figure 5I) and blood levels of non-esterified fatty acids (NEFAs) did not differ between genotypes (Figure 5I), their muscle content dramatically decreased after exercise only in TFEB KO mice (Figure 5J). Importantly, ketones did not differ between TFEB KO and controls (Figure 5K). These findings confirm a change in metabolic flexibility in the absence of TFEB that forced muscle cells to use lipids for ATP production. The exhaustion of the lipid fuel in KO mice results in inability to maintain the same exercise intensity of controls.

**TFEB Controls Metabolic Flexibility and Energy Balance Independently of Autophagy**

We and others have found that autophagy is important for mitochondrial quality control and is activated by exercise to clear dysfunctional mitochondria (Lo Verso et al., 2014). Thus, we checked whether TFEB controls autophagy in adult skeletal muscles. Surprisingly, TFEB activation was not sufficient to enhance autophagy flux and TFEB deletion did not impair autophagy flux in the presence or absence of nutrients (Figures S6A and S6B). Moreover, activation of TFEB did not induce protein breakdown and muscle loss. In fact, most of the atrophy-related genes belonging to the ubiquitin proteasome and autophagy-lysosome systems were not induced by TFEB expression (Figure S6C). Similarly, mitophagy genes were not upregulated. Since protein degradation is not affected by TFEB activation, we checked whether genes related to protein synthesis were modulated by TFEB. However, when we checked a cross-sectional area, we found a shift toward smaller size in transgenic muscles, suggesting that protein synthesis was not induced. This decrease in fiber size is due to a metabolic shift because oxidative fibers are smaller than glycolytic skeletal muscle fibers (Figure S7A). Furthermore, we did not find any significant difference in myosin distribution between transgenic and control muscles (Figure S7B). Therefore, TFEB controls myofiber metabolism, but not myosin content/type, independently of autophagy or proteostasis.

**TFEB Controls Glucose Homeostasis and Insulin Sensitivity Independently of PGC1α**

Because muscles from TFEB KO mice contain lower glycogen levels than controls, we reasoned that they may have abnormal regulation of glucose homeostasis. Thus, we performed euglycemic-hyperinsulinemic (EU) clamps and observed that the glucose infusion rate (GIR) that is required to maintain a constant glyceremia during insulin treatment was significantly reduced in TFEB KO compared to control mice (Figure 5L). The reduction of GIR was consequent to a decrease in skeletal muscle glucose uptake (Figure 5M), which caused a decrease of glycogen synthesis (Figure 5N). Although there was an apparent small decrease in adipose tissue glucose uptake, this was not statistically significant (Figure 5O). Moreover, insulin was equally effective in suppressing hepatic glucose output during the clamp experiment (Figure 5P). Together, these data demonstrate that TFEB deficiency in skeletal muscle results in peripheral insulin resistance-reduced glucose uptake and decreased glycogen content.

These findings are consistent with the transcriptomic signature of TFEB-overexpressing and TFEB KO muscles. To further confirm these findings, we monitored the expression levels of glucose homeostasis-related genes in muscles injected with AAV-TFEB and in muscles of TFEB transgenic mice and found a significant increase in the expression of GLUT1 and GLUT4, the GTPase involved in GLUT4 translocation (TBC1D1), and

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**Figure 5. TFEB Controls Energy Balance in Skeletal Muscle**

(A) High-intensity exhaustive exercise. To determine exercise capacity, mice were run on a treadmill. During high-intensity exercise, transgenic mice (red) ran more, while TFEB KO mice (gray) ran half as much as WT mice (white). Data are shown as mean ± SE, n = 10; **p < 0.01.

(B and C) Energy expenditure (B) and RER (C) were determined during exercise in TFEB KO and WT mice. The mice ran at a fixed speed of 10 m/min and an incline of 20°. The figure shows the mean RER measured at peak oxygen consumption. Data were transformed by Blom’s method to obtain both normally distributed data and normally distributed residual. Two-way ANOVA was used for comparison of RERs in the two groups during the entire resting period, whereas t test was used for comparison of individual time points, n = 8.

(D–F) Blood and muscle metabolites before and after high-intensity exercise in WT (white), TFEB transgenic (red), and TFEB KO mice (gray). Data are shown as mean ± SE, n = 8; *p < 0.05, **p < 0.01, ***p < 0.001.

(G) Enzymatic quantification of muscle glycogen before and after high-intensity exercise in WT (white), TFEB transgenic (red), and TFEB KO mice (gray). Data are shown as mean ± SE, n = 8; **p < 0.01.

(H) Periodic acid-Schiff (PAS) staining of cryosections from AAV2.1-GFP, AAV2.1-TFEB transfected, and TFEB KO mice. Inserts show PAS staining after glycogen breakdown. The scale bars represent 100 μm.

(I and J) Quantitative analysis of serum (I) and muscle (J) NEFA before and after high-intensity exercise in TFEB KO (gray) and WT mice (white). Data are shown as mean ± SE, n = 8; *p < 0.05, **p < 0.01.

(K) Enzymatic quantification of B-hydroxybutyrate before and after high-intensity exercise in TFEB KO (gray box) and WT mice (white box). Data are shown as mean ± SE, n = 8; *p < 0.05, **p < 0.01.

(L) TFEB ablation results in a decreased rate of insulin-stimulated GIR. EU clamps were used to assess whole-body insulin sensitivity by determining the GIR required to maintain euglycemia in WT (white) and TFEB KO (gray) mice. Data represent the means ± SE from five to six individual mice per group; *p < 0.05.

(M) TFEB deletion results in decreased insulin-stimulated glucose uptake in muscle. Insulin-stimulated glucose uptake into muscle tissues was determined by 2-deoxy-d-[1-14C]glucose injection during the last 35 min of insulin infusion during the EU clamp. These data represent the means ± SE of five to six mice per group; *p < 0.05. WT, white; TFEB KO, gray.

(N) The amount of glucose conversion to glycogen in GCN muscles from WT (white) and TFEB KO (gray) mice was determined during the EU clamp by infusion of [3-3H]glucose. Data represent the means ± SE from five to six mice per group; *p < 0.05.

(O and P) TFEB KO mice do not show any significant difference in glucose homeostasis of liver or adipose tissue. (O) Glucose uptake into adipose tissue (EPI) and (P) insulin suppression of hepatic glucose output (HGP, liver) were determined during the EU clamp by infusion of 2-deoxy-d-[1-14C]glucose or [3-3H]glucose infusion into WT mice (white) and TFEB KO (gray) mice. These data represent the means ± SE from five to six mice per group.
the rate-limiting enzymes of glycolysis (hexokinase 1 and 2) (Figure 6A). Immunoblotting analyses confirmed increased protein levels of GLUT1 in TFEB-overexpressing muscles (Figure 6B). The induction of genes involved in glucose uptake was also coupled with an increase of transcript and protein of GYS (Figures 6C and 6D).

GYS activity is negatively regulated by phosphorylation of the C-terminal region by GYS kinases (GSK3s) (Jensen and Lai, 2009). However, the phospho-GYS levels were unchanged in AAV-TFEB muscles, and therefore, the pGYs/GYS ratio was dramatically decreased in TFEB-overexpressing muscles when compared to controls (Figure 6D). Furthermore, expression analysis of genes related to glucose metabolism did not reveal any significant difference between PGC1α KO and controls after TFEB overexpression (Figure 6E). Finally, EM showed an accumulation of glycogen in PGC1α KO mice that were infected by AAV2.1-TFEB (Figure S4B). Quantitative and qualitative analyses of glycogen showed that TFEB induced a higher glycogen accumulation in PGC1α KO muscle than in WT (Figures 6F and 6G). These data indicate that TFEB is able to control muscle glycogen content in a PGC1α-independent manner.

**Glucose-Related Signaling Pathways Are Affected by TFEB**

The activity of GLUT transporters is tightly controlled by several pathways. Thus, we checked whether TFEB impinges not only on GLUT1/4 expression but also on glucose-related signaling. Previous studies have shown that nitric oxide (NO) controls several metabolic aspects of skeletal muscle, including mitochondrial biogenesis and glucose uptake. NO is formed by nitric oxide synthase (NOS) via the conversion of L-arginine to L-citruline. Skeletal muscles express neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) isoforms (Andrew and Mayer, 1999). Moreover, nNOS is the major isoform involved in AMPK-dependent regulation of GLUT4 (Lira et al., 2007). We found that the nNOS transcription and protein expression were increased in TFEB-overexpressing muscle. Conversely, real-time PCR and immunoblotting experiments showed a decrease of nNOS expression in TFEB KO muscle, as compared with controls (Figures 7A and 7B). To test whether nNOS and glucose transporter genes are direct targets of TFEB, we analyzed their promoters and identified CLEAR sites. ChIP experiments showed that TFEB is recruited on nNOS, GLUT1, and GLUT4 promoters (Figure 7C). Since AMPK is a downstream target of nNOS (Lira et al., 2010), we monitored the activation of AMPK. TFEB overexpression in muscle showed a significant induction of AMPK phosphorylation and of its downstream target acetyl-CoA carboxylase (ACC). Interestingly, TFEB overexpression triggered protein kinase B (AKT) activation. However, no changes in pAMPK and pACC were found in TFEB KO muscles (Figure 7D). Consistent with the presence of insulin resistance, we detected decreased AKT phosphorylation in TFEB KO muscles.

**DISCUSSION**

The beneficial effects of physical activity on mitochondrial content/function, fatty acid oxidation, and glucose homeostasis are well known (Hawley, 2002; Holloszy and Coyle, 1984; Holloszy et al., 1998). Indeed, muscle activity is important to counteract disease progression in diabetes, obesity, and metabolic syndrome. Here we have found that TFEB is a major regulator of glucose homeostasis and mitochondrial biogenesis to provide the energetic support to maintain muscle contraction. Our in vivo data show that the absence of TFEB causes accumulation of morphologically abnormal and dysfunctional mitochondria that display impairment in respiratory chain complex II activity and proton leakage, leading to a defect in ATP production and exercise intolerance. Conversely, overexpression of TFEB induces mitochondrial biogenesis, improves respiratory chain complex activities, and increases ATP production. These findings are surprising since the documented effects of TFEB are mainly related to lysosomal biogenesis and autophagy regulation.

The positive effects of TFEB on the mitochondrial network in skeletal muscle appear to be independent from PGC1α. We found that TFEB is sufficient to induce the expression of NRF2 and Tfam, two major master regulators of mitochondrial biogenesis in muscle, even in the absence of both PGC1α and PGC1β, and is both sufficient and required for PPARα expression. Therefore, TFEB acts independently of PGC1α to promote oxidation of glucose and lipids, which are critical substrates during the early and late phase of strenuous contraction, respectively. Therefore, TFEB is at least one missing factor that explains why PGC1α is dispensable for exercise and mitochondrial biogenesis (Rowe et al., 2012). Moreover, TFEB directly controls glucose homeostasis via GLUT1/4 expression and insulin sensitivity via nNOS. Indeed, muscle from TFEB KO mice showed decreased glucose uptake during EU clamps, nearly completely absent glycogen stores, and reduced AKT phosphorylation under resting conditions. The insulin resistance of the TFEB-deficient fibers prevents glucose oxidation and therefore drives the exercising muscle to use fatty acid oxidation, which consequently blocked pyruvate dehydrogenase (PDH) enzyme (Figure 2A), resulting in lactate accumulation. Altogether, these findings suggest that TFEB is a critical player of metabolic flexibility during physical activity.

In a previous study, we reported that TFEB regulates lipid metabolism in liver, and this effect appears to be mediated, at least in part, by PGC1α (Settembre et al., 2013). Conversely, our findings in muscle show that TFEB is directly involved in mitochondrial function and glucose homeostasis independently from PGC1α. These observations indicate that the networks of genes regulated by TFEB are context specific, for the gene expression profiles have significant tissue-specific changes, supporting distinct tissue-specific metabolic functions.

Calcium signaling is greatly affected by exercise, and the calcium-dependent phosphatase calcineurin is one of the most important players for muscle adaptation to physical activity. We have recently found that exercise triggers TFEB nuclear localization in a calcineurin-dependent fashion (Medina et al., 2015). Calcineurine dephosphorylates TFEB serine residues that play a critical role in determining TFEB subcellular localization and promotes its nuclear translocation (Medina et al., 2015). Importantly, calcium-dependent signaling also modulates exercise-dependent, glucose-related pathways. In fact, muscle-specific transgenic mice that overexpress an activated form of calcineurin show increased glycogen accumulation and lipid oxidation (Long et al., 2007) and upregulation of PGC1α, several glycolytic enzymes, mitochondrial genes, genes related to lipid metabolism, and GLUT4 (Long and Zierath, 2008). Conversely,
Figure 6. TFEB Controls Genes Related to Glucose Metabolism

(A and C) Quantitative real-time PCR of glucose uptake and glycogen biosynthesis-related genes in GCN muscles infected with AAV2.1-TFEB (black bar) and in TFEB transgenic muscles (red bar) compared with WT (white bar) muscles. Data were normalized for GAPDH and expressed as fold induction relative to the WT. Data are shown as mean ± SE, n = 3; *p < 0.05, **p < 0.01.

(B) Western blot of GLUT1 and densitometric quantification. Values are normalized for GAPDH; *p < 0.05.

(D) Western blot analysis of total and phosphorylated GYS from extracts of GCN. Representative blot images are shown (left panel). Densitometric quantification is depicted on the right panel. Data are shown as mean ± SE, n = 3; *p < 0.05.

(E–G) TFEB regulates glycogen synthesis independently of PGC1α.

(E) Quantitative real-time PCR of genes related to glucose uptake and glycogen biosynthesis in PGC1α−/− and WT mice that were infected with AAV2.1-GFP (white) or AAV2.1-TFEB (black). Data are shown as mean ± SE, n = 3; *p < 0.05, **p < 0.01.

(F) PAS staining of cryosections from PGC1α−/− muscles that were transfected by AAV2.1-GFP or AAV2.1-TFEB. The scale bars represent 100 μm.

(G) Enzymatic quantification of basal glycogen levels in PGC1α−/− and WT muscle, infected with AAV2.1-GFP (white) or AAV2.1-TFEB (black).
Muscle-specific calcineurin KO mice show exercise intolerance when subjected to exhausting physical activity (Pfluger et al., 2015). Finally, immunosuppression therapy by high-dose treatment with calcineurin inhibitors cylosporin A or tacrolimus (FK-506) has been associated with a higher risk for developing obesity and diabetes in patients (T D Correia et al., 2003). Altogether, these data suggest that calcineurin plays a major role in glucose and lipid metabolism, although the mechanistic insights of these beneficial effects of calcineurin are unknown.

Our data suggest that TFEB is a critical calcineurin downstream target that coordinates metabolic adaptations such as glucose uptake and mitochondria function to optimize energy production to sustain muscle contraction.

TFEB not only regulates expression of glucose transporters and critical glycolytic enzymes but also factors that impinge on AMPK regulation, such as nNOS. It was shown that nNOS controls GLUT4 expression in skeletal muscle cells through AMPK activation. In addition, endogenous nNOS is required for the upregulation of AMPK activity by the AMP mimetic, AICAR (Higaki et al., 2001; Lira et al., 2010; McConell et al., 2010).

In summary, our results position TFEB as a central coordinator of insulin sensitivity, glucose homeostasis, lipid oxidation, and...
mitochondrial function, further emphasizing the importance of this regulatory pathway in the metabolic response to energy-demanding conditions such as physical exercise.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Plasmids, and Transfection Reagent**
Detailed information in the Supplemental Experimental Procedures.

**Real-Time PCR**
Quantitative real-time PCR analyses were performed on a LightCycler 480 II (Roche). For detailed information on preparation and gene expression analysis, see the Supplemental Experimental Procedures.

**Generation of Muscle-Specific TFEB KO and Inducible Muscle-Specific Transgenic Mice and In Vivo Experimental Procedures**
Detailed information in the Supplemental Experimental Procedures.

**Western Blot Analysis and Antibodies**
Total homogenates were prepared in RIPA buffer (50 mM Tris HCl [pH 8], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) with the addition of a protease inhibitor cocktail (Roche). Protein concentration was determined by the Lowry method. Aliquots, 50 μg each, were run through an SDS-PAGE and electrophobetted onto a PVDF membrane, which was then matched with different antibodies. For the antibodies used in western blot analysis, see the Supplemental Experimental Procedures.

**Biochemical Analysis of Mitochondrial Respiratory Chain Complex**
Morphological Analysis
Histochemical and ultrastructural analyses were performed as described (Sciacco and Bonilla, 1996). Detailed information in the Supplemental Experimental Procedures.

**Isolation of Skeletal Myofibers and Measurements of Mitochondrial Membrane Potential**
Muscle fibers were isolated from FDB muscle and mitochondrial membrane potential measured by epifluorescence microscopy on the basis of the accumulation of TMRM fluorescence, as previously described (Lo Verso et al., 2014). Fibers were considered as depolarizing when they lost more than 10% of their initial value of TMRM fluorescence. Imaging was performed with a Zeiss Axiovert 100 TV inverted microscope equipped with a 12-bit digital cooled charge-coupled device camera (Micromax, Princeton Instruments). The results were analyzed with MetaFluor imaging software (Universal Imaging).

**Acute Exercise, Training, and Fatigue Experiments**
For acute and chronic exercise studies, 16-week-old mice performed concentric exercise on a treadmill (Biological Instruments, LE 8710 Panlab Technology 2B) with a 10° incline, according to the protocol of exercise previously described (Medina et al., 2019). Total running distance was recorded for each mouse. Detailed information in the Supplemental Experimental Procedures.

**Immunohistochemistry**
Detailed information in the Supplemental Experimental Procedures.

**Electron Microscopy**
Detailed information in the Supplemental Experimental Procedures.

**Plasma Chemistry Analysis**
Blood was collected from the orbital plexus under isoflurane (Vedco) anesthesia. Plasma was frozen in aliquots at −20°C or used immediately after collection. Specific enzymatic kits were used for determination of serum NEFAs (Wako) and lactate (Abcam). Plasma glucose was monitored by a glucometer. Insulin was measured by ELISA (Mericodia).

**Tissue Metabolite Quantification**
Detailed information in the Supplemental Experimental Procedures.

**Whole-Body Indirect Calorimetry**
Metabolic measurements were performed using an Oxymax indirect calorimetry system (Columbus Instruments) (Zong et al., 2011). Detailed information in the Supplemental Experimental Procedures.

**In Vivo Assessment of Insulin Action and Glucose Metabolism**
Four days before the experiment, the mice were anesthetized and an indwelling catheter was introduced into the left internal jugular vein. The mice were fully recovered from the surgery before the in vivo experiments, as reflected by their reaching preoperative weight. After an overnight fast, EU clamps were conducted in conscious mice as previously described (Ayala et al., 2006; Zong et al., 2012). Detailed information in the Supplemental Experimental Procedures.

**ChIP Assays**
We performed ChIP assays on adult skeletal muscle overexpressing TFEBx3-Flag, and on WT muscle as control, by using the ChIP assay kit (Upstate) according to Milan et al. (2015). For immunoprecipitation, we used anti-FLAG antibody (F7425, Sigma-Aldrich). Oligonucleotide primers for amplification of a TFEB binding site on the GLUT1, GLUT4, nNOS, NRF1, NRF2, and TFAM promoters are listed in Table S7.

**Microarray Data Analysis**
Detailed information in the Supplemental Experimental Procedures.

**Statistical Analysis**
Data are expressed as mean values ± SE. Results were evaluated by repeated-measures ANOVA, multivariate ANOVA (MANOVA), or Student’s two-tailed t test. p < 0.05 was considered statistically significant. In all figures, *p < 0.05, **p < 0.01, and ***p < 0.001.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes Supplemental Experimental Procedures, seven figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2016.11.003.

**AUTHOR CONTRIBUTIONS**
G.M., M.S., and A.B. designed the project and experiments and wrote the manuscript. G.M., A.A., and C.V. performed experiments and analyzed and interpreted data. G.M. and A.A. generated transgenic mice, E.V.P., C.L., I.D.M., V.R., H.Z., B.B., F.S., and C.T. contributed to experiments and data collection; P.G. and P.B. provided reagents and discussed data; R.D.C. performed computational analysis; L.D., S.M., and P.K.S. provided technical expertise. J.E.P., M.Z., M.S., and A.B. discussed, reviewed, and edited the manuscript. M.S. and A.B. supervised the work.

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Supplemental Information

Transcription Factor EB Controls
Metabolic Flexibility during Exercise

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Figure S1.

A

TFEB Expression

Fold Activation

WT

AAV-TFEB

B

TFEB Expression

Relative Expression

WT

TFEB-KO

Gastrocnemius   Quadriceps       Diaphragm            Heart                 Liver               Kidney
Figure S1. Expression analysis of TFEB transcript. Related to Figure 1. (A) The TFEB transcript from AAV2.1-TFEB transfected muscle was quantified by qRT-PCR (B) Expression levels of endogenous Tcfeb mRNA in muscles isolated from TFEB-KO and WT mice. Bar represent mean ± SE for n = 5; **p<0.01, ***p<0.001s.
Figure S2.

A

DsRed/MITO  Dapi  DsRed/MITO

GFP

TFEB/GFP

B

Empty Vector  TFEB

C MtDNA/C2C12

D

TFEB  TFAM  NRF1  NRF2

COX1  COX2  COX4  COX5a

PGC1α  PGC1β
Figure S2. TFEB induces mitochondrial biogenesis in C2C12 cells. Related to Figure 1. (A) Confocal images of C2C12 cells co-transfected with DsRed/MITO and with CMV-GFP or TFEB-GFP. Nuclei were stained with DAPI (blue). Mitochondria are shown in red. DAPI and DsRed were excited with 405 (UV) and 561 laser lines, respectively. Images were acquired on a Perkin Elmer Opera automated confocal microscope. The scale bars represent 10 µm. Percentage of DsRed/MITO intensity was calculated using Prism software (see Materials and Methods for details) Mean ± SE, n = 3; **p<0.01. (B) Electron microscopy analysis of C2C12 cells that overexpress TFEB-3XFlag compared with control cells. The scale bars represent 500 nm. (C) Quantitative MtDNA analysis, by Rt-PCR, confirmed an increase of mtDNA when TFEB is overexpressed in muscle cells compared with WT condition. Error bars represent mean ± SE for n=3; **p<0.01. (D) Expression analysis of genes related to mitochondrial biogenesis and functions in C2C12 cells transfected by empty vector (white bar) or TFEB-3XFlag (black bar). Error bars represent mean ± SE for n=3; *p<0.05, **p<0.01, ***p<0.001.
Figure S3.

(A) WT TFEB tg

SDH

WT

TFEB tg

(B) Glut/Mal Succ/Rot

WT TFEB tg

**

nmolO2/min/mg protein

(C) Protein carbonylation

WT TFEB-KO WT TFEB-KO

Exercise

WT TFEB-KO WT TFEB-KO

**

Protein carbonylation

Exercise
Figure S3. TFEB increases respiratory chain activity and glycogen synthesis in skeletal muscle. Related to Figure 2. Succinate dehydrogenase (SDH) staining of cryosections from TFEB transgenic mice compared with control mice. TFEB was induced for 2 weeks in 5 months old mice. The scale bars represent 100 nm. (B) Respiration of mitochondria from muscles of TFEB transgenic and control mice. TFEB significantly increased oxygen consumption rate when glutamate/malate were used as substrates. Data are presented as mean ± SE; n=3; **p < 0.01. (C) Overall protein carbonylation of WT and TFEB-KO muscles revealed by Oxyblot. A representative immunoblot for carbonylated proteins is depicted on the left; densitometric quantification of the carbonylated proteins is shown in the graph on the right. TFEB- KO mice show higher carbonylated than WT in both sedentary condition and after exercise. n=4; *p < 0.05). Values are mean ± SE for n = 4; *p < protein 0.05.
Figure S4.

A. 

| PGC1α−/− | PGC1α−/− Exercise |
|---------|------------------|
| Control| Anti- | Anti-TFEB | Hematoxylin Staining |
|       |        |          |                   |

B. 

AAV-TFEB 

PGC1α−/− 

C. 

AAV-GFP | AAV-TFEB 

COX | SDH 

D. 

| Citrate Synthase | Complex I |
|------------------|-----------|
|                  | AAV-GFP   | AAV-TFEB |
| Specific Activity (%) | WT | PGC1α−/− | WT | PGC1α−/− |

| Complex II | Complex III | Complex IV |
|-----------|-------------|-----------|
| Specific Activity (%) | WT | PGC1α−/− | WT | PGC1α−/− | WT | PGC1α−/− |
Figure S4. Exercise induces TFEB expression and nuclear localization in PGC1α−/− muscle. Related to Figure 3. (A) TFEB immunohistochemical analysis of PGC1α−/− muscles from sedentary and exercised mice. The muscles were infected by intramuscular injection of AAV2.1 control and AAV2.1-TFEB virus. Gastrocnemius muscle cryosections were immunostained by using anti-TFEB antibody and counter-stained with hematotoxylin for nuclei detection. Control means endogenous TFEB in muscles infected with control virus. TFEB means the transgene TFEB in muscles infected with AAV-TFEB. Arrows indicate exercise-induced TFEB nuclear localization. The scale bars represent 100 nm. (B-D) PGC1α is not required for mitochondrial biogenesis induced by TFEB. (B) EM of PGC1α−/− muscles that were infected with AAV2.1-TFEB or AAV2.1-GFP. The electron micrographs reveal an accumulation of mitochondria in TFEB-overexpressing muscle and a granular staining consistent with glycogen b-particles. The scale bars represent 500 nm. (C) COX and SDH staining of cryostatic sections from PGC1α−/− muscles transfected by AAV2.1-GFP or AAV2.1-TFEB. The scale bars represent 100 nm. (D) The mitochondrial respiratory chain activity in control and TFEB-overexpressing muscles. Data are expressed as percentage respect to the WT muscles infected with AAV-GFP control vector. Error bars represent mean ± SE for n=3; *p<0.05.
Figure S5.

A

**PGC1β**

![Fold Activation](image)

**TFAM**

![Fold Activation](image)

**NRF1**

![Fold Activation](image)

**NRF2**

![Fold Activation](image)

B

**PGC1α**

![Fold Activation](image)

**PGC1β**

![Fold Activation](image)

**TFAM**

![Fold Activation](image)

**NRF1**

![Fold Activation](image)

**NRF2**

![Fold Activation](image)

**COX1**

![Fold Activation](image)

**COX4**

![Fold Activation](image)

**COX5a**

![Fold Activation](image)

**COX8a**

![Fold Activation](image)
Figure S5. TFEB controls mitochondrial biogenesis and activity by a PGC1α and PGC1β-independent mechanism. Related to Figure 3. (A) Expression analysis of genes related to mitochondrial biogenesis in WT and PGC1α/- muscles that were transfected by AAV2.1-GFP (white bar) or AAV2.1-TFEB (black bar). Error bars represent mean ± SE for n=3; *p<0.05, **p<0.01, ***p<0.001. (B) Expression analysis of genes related to mitochondrial biogenesis and mitochondrial respirator chain in Hela cells silenced for PGC1α PGC1β and transfected with TFEB or empty vector. Error bars represent mean ± SE for n=3; *p<0.05, **p<0.01, ***p<0.001.
Figure S6.

A

|       | WT | TFEB tg |
|-------|----|---------|
| Colch.| -  | +       |

- p62
- LC3 I
- LC3 II
- GAPDH

B

|       | WT | KO      |
|-------|----|---------|
| fed   | -  | -       |
| stv24 | +  | +       |

- LC3 I
- LC3 II
- GAPDH

C

TFEB

- Fold Activation

+****

Autophagy and Mitophagy

- Fold Activation

+*

Ubiquitin Ligases

- Fold Activation

+****

Protein Synthesis

- Fold Activation

+**
Figure S6. Autophagy flux is not changed by TFEB-overexpression or deletion. Related to Figure 5. (A) Upper panel: representative blots of p62 and LC3 from protein lysates of gastrocnemius muscles of WT and TFEB transgenic (tg) animals. Mice were treated with colchicine (Colch.) or vehicle for autophagy flux measurements. The densitometric quantification is depicted on the right panel. Lower panel: immunoblot analysis of LC3 from protein lysates of gastrocnemius muscles from fed or 24 h fasted TFEB tg or control mice. GAPDH was used as loading control. (B) Representative blot and densitometric quantification of LC3 from protein lysates from gastrocnemius muscles of fed and starved TFEB-KO or controls mice. Mice were treated with colchicine or vehicle for autophagy flux measurements. GAPDH was used as loading control. (C) Quantitative RT-PCR of TFEB, autophagy/mitophagy (p62/SQSTM1, LC3, Pink, Parkin, Bnip3 and Gabarapl), atrophy-related ubiquitin ligases (MusA1, Atrogin1, MuRF1, Fbxo31, Fbxo21 and Itch) and protein synthesis (S6, mTOR and 4EBP1) transcripts. Data are representative of four independent experiments. Data are normalized to GAPDH and expressed as fold increase of control animals. n=4 muscles in each group Values are mean ± SE *p<0.05, ***p<0.001, ****p<0.0001.
Figure S7.

(A) Cross-sectional area (µm²)

(B) Gastrocnemius Fiber type %

WT

TFEB tg

Type I

Type IIA

Type IIX+IIB
Figure S7. TFEB-overexpression mouse muscles modifies fiber size but does not affect MHC expression. Related to Figure 5. (A) Frequency histograms showing the distribution of myofibers cross-sectional areas (μm$^2$) from gastrocnemius muscles of inducible muscle-specific TFEB transgenic (black bars) and control male mice (white bars). (B) Average cross-sectional area of myofibers from gastrocnemius muscles of TFEB tg and control male mice. Values are shown as means ± SE *p<0.05, from 4 muscles in each group. Percentage of type I and type II fibers in WT and TFEB tg was calculated using ImageJ 1.47 software (see Materials and Methods for details). Values are shown as means ± SE *p<0.05, from 3 muscles in each group. (C) Cryosections of Gastrocnemius muscle were immunostained for the different MHC I, MHCIIa, and MHCIIb myosins. Type I (blue), type IIA (green), type IIB (red) and type IIX (unstained) fibers are depicted. The scale bars represent 100 nm.
Supplemental Tables

Table S1. TFEB overexpression muscle dataset. Related to Figure 1. Complete list of differentially expressed genes in TFEB overexpression muscle microarray dataset (GSE62975). The genes significantly (FDR<0.05; FoldChange ≥1.5) up-regulated and down-regulated are highlighted in red and in green, respectively.
(supplied as Excel file: Supplemental Table 1.xlsx).

Table S2. TFEB-KO muscle dataset. Related to Figure 1. Complete list of differentially expressed genes in TFEB-KO muscle microarray dataset (GSE62976). The genes significantly (FDR<0.05) up-regulated and down-regulated are highlighted in red and in green, respectively.
(supplied as Excel file: Supplemental Table 2.xlsx).

Table S3. TFEB controls lipid and glucose metabolism in muscle. Related to Figure 1. List of Lipid and Glucose Metabolism related terms to the GOEA induced by TFEB overexpression and inhibited by TFEB-KO. For each term the genes significantly induced by TFEB overexpression (Supplemental Table BPALL_UP_OverTFEB) and inhibited by TFEB-KO (Supplemental Table BPALL_DOWN_KO-TFEB) are reported in table.
(supplied as Excel file: Supplemental Table 3.xlsx).

Tables S4. Mitochondrial genes up-regulated in TFEB overexpression muscle. Related to Figure 1. List of 38 genes significantly up-regulated in TFEB overexpression muscle dataset, with a main localization in the mitochondrion.

| Gene Symbol | Gene Title |
|-------------|------------|
| Abat        | 4-aminobutyrate aminotransferase |
| Arl2bp      | ADP-ribosylation factor-like 2 binding protein |
| Bak1        | BCL2-agonist/killer 1 |
| Bik         | BCL2-interacting killer |
| Bid         | BH3 interacting domain death agonist |
| Cds2        | CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 2 |
| Dna2        | DNA replication helicase 2 homolog (yeast) |
| Htra2       | HtrA serine peptidase 2 |
| Rab11fip5   | RAB11 family interacting protein 5 (class I) |
| Acs6        | acyl-CoA synthetase long-chain family member 6 |
| Aldh18a1    | aldehyde dehydrogenase 18 family, member A1 |
| Alas1       | aminolevulinic acid synthase 1 |
| Cpt1a       | carnitine palmitoyltransferase 1a, liver |
| Cidea       | cell death-inducing DNA fragmentation factor, alpha subunit-like effector A |
| Ckm1        | creatine kinase, mitochondrial 1, ubiquitous |
| Cyb5r3      | cytochrome b5 reductase 3 |
| Cox6a1      | cytochrome c oxidase, subunit VI a, polypeptide 1; predicted gene 7795 |
| Gyk         | glycerol kinase |
| Gatm        | glycine amidinotransferase (L-arginine:glycine amidinotransferase) |
| Hspa1b      | heat shock protein 1B; heat shock protein 1A; heat shock protein 1-like |
| Hk1         | hexokinase 1 |
| Me2         | malic enzyme 2, NAD(+)-dependent, mitochondrial |
| Mstol       | misato homolog 1 (Drosophila) |
| Mrpl39      | mitochondrial ribosomal protein L39 |
| Maa1        | monoamine oxidase A |
| Nos1        | nitric oxide synthase 1, neuronal |
| Ppif        | peptidylprolyl isomerase F (cyclophilin F) |
| Gene Symbol | Gene Title                                                                 |
|-------------|---------------------------------------------------------------------------|
| Pmaip1      | phorbol-12-myristate-13-acetate-induced protein 1                          |
| Sfxn1       | sideroflexin 1                                                            |
| Ugt1a9, Ugt1a2, Ugt1a6a, Ugt1a7c, Ugt1a10, Ugt1a1, Ugt1a5, Ugt1a6b | UDP glucuronosyltransferase 1 family                                     |
| Cox7c       | similar to cytochrome c oxidase, subunit VIIc; predicted gene 3386         |
| Slc25a5     | solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 5; similar to ADP/AMP translocase 2 (Adenine nucleotide translocator 2) (ANT 2) (ADP,ATP carrier protein 2) |
| Slc25a24    | solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 24 |
| Slc25a36    | solute carrier family 25, member 36                                       |
| Slc25a37    | solute carrier family 25, member 37                                       |
| Slc25a45    | solute carrier family 25, member 45                                       |
| Tmihc       | trimethyllysine hydroxylase, epsilon                                       |
| Tdrd7       | tudor domain containing 7                                                |

Table S5. Mitochondrial genes down-regulated in TFEB-KO muscle. Related to Figure 1. List of 73 genes significantly inhibited in the TFEB-KO muscle dataset, with a main localization in the mitochondrion.
| Gene   | Description |
|--------|-------------|
| Fth1   | ferritin heavy chain 1 |
| Gatm   | glycine amidinotransferase (L-arginine:glycine amidinotransferase) |
| Gcdh   | glutaryl-Coenzyme A dehydrogenase |
| Gfm2   | G elongation factor, mitochondrial 2 |
| Got2   | glutamate oxaloacetate transaminase 2, mitochondrial |
| Gpam   | glycerol-3-phosphate acyltransferase, mitochondrial |
| Gpd1   | glycerol-3-phosphate dehydrogenase 1 (soluble) |
| Gpd2   | glycerol phosphate dehydrogenase 2, mitochondrial |
| Ide    | insulin degrading enzyme |
| Idh2   | isocitrate dehydrogenase 2 (NADP+), mitochondrial |
| Idh3a  | isocitrate dehydrogenase 3 (NAD+) alpha |
| Imm1   | inner membrane protein, mitochondrial |
| Isca1  | iron-sulfur cluster assembly 1 homolog (S. cerevisiae) |
| Ivd    | isovaleryl coenzyme A dehydrogenase |
| Ldhb   | lactate dehydrogenase B; predicted gene 5514 |
| Mavs   | mitochondrial antiviral signaling protein |
| MccC2  | methylcrotonoyl-Coenzyme A carboxylase 2 (beta) |
| Mdh2   | malate dehydrogenase 2, NAD (mitochondrial) |
| Mfn1   | mitofusin 1 |
| Mrrl15 | mitochondrial ribosomal protein L15 |
| Myl10  | myosin, light chain 10, regulatory |
| Ndua10 | NADH dehydrogenase (ubiquinone) 1 alpha |
| NduFlv1| NADH dehydrogenase (ubiquinone) flavoprotein 1 |
| LOC100045796, Nfs1 | nitrogen fixation gene 1 (S. cerevisiae) |
| Nos1   | nitric oxide synthase 1, neuronal |
| Nudt19 | nudix (nucleoside diphosphate linked moiety X)-3-oxoacid CoA transferase 1 |
| Oxtc1  | presenilin associated, rhomboid-like |
| Pdk2   | pyruvate dehydrogenase kinase, isoenzyme 2 |
| Pln    | phospholamban |
| Pnkd   | paroxysmal nonkinesiogenic dyskinesia |
| Ppif   | peptidylprolyl isomerase F (cyclophilin F) |
| Prdx6  | Peroxiredoxin-6 (Antioxidant protein 2) |
| Pxmp2  | peroxisomal membrane protein 2 |
| Rk     | riboflavin kinase |
| Rhot2  | ras homolog gene family, member T2 |
| Rtn4ip1| reticulon 4 interacting protein 1 |
| Sdhb   | succinate dehydrogenase complex, subunit B, iron |
| Arts (Sept4) | septin 4 |
| Shmt1  | serine hydroxymethyltransferase 1 (soluble) |
| Slc25a15| solute carrier family 25 (mitochondrial carrier) |
| Suclg2 | succinate-Coenzyme A ligase, GDP-forming, beta |
| Supv31l| suppressor of var1, 3-like 1 (S. cerevisiae) |
| Tmem186| transmembrane protein 186 |
| Tuflm  | predicted gene 9755; Tu translation elongation |
| Ung    | uracil DNA glycosylase |
| Uqcre1 | ubiquinol-cytochrome c reductase core protein 1 |
Table S6. CLEAR sites on mitochondrial genes. Related to Figure 2. Position and sequence of the CLEAR sites (i.e. the consensus TFEB binding-site) in the promoter region (1Kb upstream the TSS in the human and Mouse promoters) of mitochondrial genes down-regulated in TFEB-KO muscle. (supplied as Excel file: Supplemental Table 6.xlsx).

Table S7. CLEAR sites in promoter region of genes related to mitochondria biogenesis and glucose metabolism. Related to Figure 2 and Figure 7. Position and sequence of TFEB binding site on the GLUT1, GLUT4, nNOS, TFAM, NRF1 and NRF2 promoter. (supplied as Excel file: Supplemental Table 7.xlsx).
**Supplemental Experimental Procedures**

**Cell culture, Plasmids and Transfection Reagent.**
C2C12 and HeLa cells were purchased from ATCC and cultured in DMEM media supplemented with 10% fetal bovine serum, 200 μM L-Glutamine, 100 μM sodium pyruvate, in 5% CO₂ at 37°C. Cells were transfected with pDsRed2-Mito (Clontech) and Human full-length TFEB-GFP or TFEB3XFlag, both constructs were previously described (Martina et al., 2012; Sardiello et al., 2009). Cells were transfected using TransIT®-LT1 Transfection Reagent (MirusBio) according to the protocol from the manufacturers. Cells were silenced with PGC1α siRNA oligonucleotides (Thermo Dharmacon, L-005111-00), PGC1β siRNA oligonucleotides (Thermo Dharmacon, L-008556-00) or non-targeting siRNAs (Thermo Dharmacon, D-001810-10-05), by direct or reverse transfection, using Lipofectamine RNAiMAX reagent (Invitrogen) according to the protocol from the manufacturer. siRNA-transfected cells were collected after 48 h, if not otherwise stated.

**Real-time PCR.**
For mtDNA content analysis, SYBR Green real-time PCR was performed using primers specific to a mouse mtDNA region in the ND1 gene and primers specific to RNaseP, a single copy gene taken as a nuclear gene reference, as described (Viscomi et al., 2009). For the analysis of transcripts, total RNA was extracted from liquid nitrogen snap frozen muscle by Trizol, according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Of total RNA, 2 μg was treated with RNase free-DNase and retrotranscribed using the ‘‘cDNA cycle’’ kit (QuantiTect Reverse Transcription Kit - QIAGEN). Approximately 2–5 ng of cDNA was used for real-time PCR assay (SYBR® Green PCR Master Mix Applied Biosystem) using primers specific for amplification of several genes. Autophagic, Lipid Metabolism and Glucose Metabolism gene-specific primers are listed below. Fold change values were calculated using the ΔΔCt method. An unpaired t-test was used to calculate statistical significance.

**List of murine primers for RT-PCR**

| Gene Name     | Primer Sequence                      |
|---------------|--------------------------------------|
| TCFEB F       | GCAGAAGAAAGACAACTCACAA               |
| TCFEB R       | GCCTTGGGATCAGCATTT                   |
| GLUT1 F       | AGCAGCAAGAGGATGGACG                  |
| GLUT1 R       | CACGGAGAGAGACAAAGC                   |
| GLUT4 F       | CCGGCGCTCTGTTAGAGATTACT             |
| GLUT4 R       | AGGCACCCCCGAGATGAGT                 |
| NOS1 F        | CCAAAGCAGAGAGATGAAAGACACA           |
| NOS1 R        | TCTTTGTAAGGACTGTGTC                  |
| PGC1alpha F   | AGCCGTGACCGAGTAAAGAG                |
| PGC1alpha R   | GCTGCAATGGTCTAGTGCTTAG              |
| GAPDH F       | TGCAACCACAACTGCTTAC                 |
| GAPDH R       | TCTTCTGGGTGGCAGTGGT                 |
| TBC1D1 F      | GTCCCCGGGTAATAAGC                   |
| TBC1D1 R      | TTGTCAACCGATGGACAGCT                |
| PGC1-beta F   | ACTGAAAGAGGCCCGAGCA                 |
| PGC1-beta R   | ATGGAAGGAGGCTGTGTCGA                |
| PPAR-alpha F  | CTTACGCTGGGAGATGAGG                 |
| PPAR-alpha R  | CCCCATTTCGGTAGAGGGTA                |
| DLAT F        | TGGTTCTCATCGTGGTTGAGTCTG            |
| DLAT R        | TGCTAATGGATGCTGCCCTTGTC             |
| CRAT F        | GGAGAAGAGAGCGAGCTCAG               |
| CRAT R        | AGATAATCCTCCACCCGCTG               |
| Gene   | Sequence          |
|--------|-------------------|
| CPT1b F | GACAGTACCCCTCCTCCACCC |
| CPT1b R | ACCAGCAAGAACAGGGAATAGGA |
| CS F    | GCCCTCGCTATTCCTTGCA |
| CS R    | AGTTCACTTCGGTCACTGCA |
| COX8B F | CGAAGTTACAGTTGCTCCC |
| COX8B R | GCTGGAAACATGAAAGCCAC |
| MDH2 F  | TGAACGGGAAAGGAGGAGTC |
| MDH2 R  | AATGCCCAAGGTTCTTCCTCCA |
| CYC1 F  | GGCTCTCCTCCTACACAG |
| CYC1 R  | CTGACCATTTATGCGGCTTC |
| ATP5A1 F | GACGTACCCCTCCTCCACCC |
| ATP5A1 R | ACCAGCAAGAACAGGGAATAGGA |
| SDHA F  | TTACCTGGTTTTTCCCTCAT |
| SDHA R  | AAGTCTGCGCAACACTAC |
| NRF1 F  | CCCACTCCGCTGCTGACT |
| NRF1 R  | CAGACACGTTTGGCTCAGGAA |
| NRF2 F  | GATCAGGGCAGCATGGTTAAGGT |
| NRF2 R  | AGAGCCCAAGACACCCCTTCC |
| HK2 F   | TGGGTTCACCTTCTCGTTC |
| HK2 R   | TGGACCTGGAACCCCTTAGTCC |
| HK1 F   | TGCTAGGCCCAGACTAC |
| HK1 R   | TTGGTTCGTAGTCTGAG |
| COX1 F  | TGCTAGGCCCAGACTAC |
| COX1 R  | CAGGGATCAGAAGTTGGTGTTT |
| COX2 F  | CAGGGCCAGTAAATCATGAA |
| COX2 R  | GAGCATGCGCATAGAATAATCTC |
| COX4 F  | TTCCTGCGCTCGTTCTGAT |
| COX4 R  | CATCGAAGATATGGAGGATG |
| COX5a F | TCATCCAGGAACCTTACAGCAAG |
| COX5a R | ACATCTGGAGGACCTTCTAG |
| TFAM F  | AGATATGCGTTGCGCCCTTG |
| TFAM R  | AAGGCTCGGCACTTCTTTG |
| CD36 F  | CCAGATGAGATGACATGACAGAT |
| CD36 R  | GTGACCTGCAGTGTTTTC |
| ND1 F   | CATAAGCTGCGTGAATCA |
| ND1 R   | CCTGTTGGTTGAGTAGT |
| RNasIP F | GCCCTACCTGGAGTCCGCTACT |
| RNasIP R | CTGACACACACAGCTGTAGAAA |
| PPAR gamma F | CGTGCACTAAGCTATGTGA |
| PPAR gamma R | GGGTGGAGCATTCTCTCGTAA |
| PPAR delta F | ATGGGGGACACAAACAC |
| PPAR delta R | GAGGAATTTCTGGGAGAGGT |
| GYS F   | TGGGAAGACTTGAGGAGATGA |
| GYS R   | CATTCCATCCCTGTACCCCT |
| PYGM F  | CTACAGAAAGGACCACAG |
| PYGM R  | CATGTGTCCTTGCGATGTC |
| Atrogin F | GCAACACTGGCCACTTCTC |
| Atrogin R | CTTAGGGAAAAGGTGAAGCG |
| Murf1 F  | ACCCTGCTGGAAAAACCATC |
| Murf1 R  | ACCCTGCTGGAAAAACCATC |
| Gene     | Forward Primer | Reverse Primer |
|----------|----------------|----------------|
| Fbxo31 F | GTATGGGGTTTGTGAGAAC |                        |
| Fbxo31 R | AGCCCCAAAATGTGTGTA |                        |
| Fbxo21 F | TCAATAAACCTCAAGGGTGTC |                        |
| Fbxo21 R | GTTTTGACACAAAAGCTCCA |                        |
| ITCR F  | CCAACCACCCACGAAGACC |                        |
| ITCR R  | CTAGGGCCGAGCCTCCAAG |                        |
| p62 F   | CCCAGTGTCTTGGGATTC |                        |
| p62 R   | AGGGAAAGCAGAGGAGCTC |                        |
| LC3 F   | CAAGCTCTCTGTATGTAGGTG |                        |
| LC3 R   | TGGTTGGGCTTTATTAGCTC |                        |
| PINK F  | ATGCTGAGGCTGAGGTTCA |                        |
| PINK R  | CGGATGATGTTAGGGTGAG |                        |
| Parkin F| CCTCTTGTGGGAGGCA |                        |
| Parkin R| GCCTGTGGCTGTTGATCA |                        |
| BNIP3 F | TTGCACTGACACTTCTGATGA |                        |
| BNIP3 R | GAACACCGCATTTCAGAAGC |                        |
| GabarapL F | CATCGTGGAGAAGGCTCCTA |                        |
| GabarapL R | ATACACGTGGCCATGTTAG |                        |
| mTOR F  | GAGAAAGGATATGAGATGAG |                        |
| mTOR R  | CCATGAGGCTTCTTGAGTA |                        |
| S6 F    | AGCCAGGACAAACGACCCAA |                        |
| S6 R    | TCCCTGGGGCTTTCTGCTTCCTC |                        |
| MUSA1 F | TCGTGGAATGTAATCTC |                        |
| MUSA1 R | CCTCCGTTTCTCTACAG |                        |

**List of human primers for RT-PCR**

| Gene     | Forward Primer | Reverse Primer |
|----------|----------------|----------------|
| PGC1 alpha F | TAGTCCTCTCTCCATGCTTG |                        |
| PGC1 alpha R | TGCAATGGTTCTGGGTACTGA |                        |
| PGC1 beta F | CTCTTCCACCTGGCACTCC |                        |
| PGC1 beta R | ACCCTGCACTTCTCATACTC |                        |
| TFAM F   | TTGAAGAGAATGGCCGAGGT |                        |
| TFAM R   | TTCTTTATATACCTGGCACTC |                        |
| NRF1 F   | CTTGTACACATCAGACTGGTA |                        |
| NRF1 R   | ACCGCAGAAATATCCATCGGG |                        |
| NRF2 F   | GCCCTGGTTAGTACACGG |                        |
| NRF2 R   | GTTTTGCTTCTGAGCTTTGA |                        |
| COX1 F   | ACCCTAGACAAACCTACGC |                        |
| COX1 R   | TGATATGACCTGGGTAGCTCC |                        |
| COX4 F   | CAAACGCAGAATTTCCACCT |                        |
| COX4 R   | GTCACGCGATCCATATAAGC |                        |
| COX5a F  | TGCCCTGGGAATTTGGTAAAG |                        |
| COX5a R  | ATTTAACCCTGCTGATGCC |                        |
| COX8a F  | GAGGGAAAGCTGGAGCAT |                        |
| COX8a R  | CCTGTAGGCTCCAGGTGTG |                        |
| ND1 F    | CCTAAACCCCCACCATCTA |                        |
| ND1 R    | GCCTAGGTTGAGGTACCA |                        |
**Generation of muscle-specific TFEB Knockout and inducible muscle-specific transgenic mice and in vivo experimental procedures.**

Mice bearing Tcfeb-flxed alleles (Tcfeb\(^{f/f}\)) (Settembre et al., 2012) were crossed with transgenic mice expressing Cre either under the control of a Myosin Light Chain 1 fast promoter (MLC1f-Cre) (Bothe et al., 2000). Genomic DNA isolated from Tcfeb\(^{f/f}\) mice was subjected to PCR analysis. Cre-mediated recombination was confirmed by PCR with genomic DNA from gastrocnemius muscles using the primers Cre forward: 3’-CACCAGGCTATCAAACG-5’ and Cre reverse: 3’-TTACATTGGTCC AGCCACCCAG-5’.

The Pgc1α Knockout mouse line was obtained from Jackson Laboratory (Bruce Spiegelman, Dana-Farber Cancer Institute) (Lin et al., 2004). The muscle-specific Pgc1α Knockout mouse was generated by crossing the Pgc1α floxed mice from Jackson Laboratory (Bruce Spiegelman, Dana-Farber Cancer Institute) with MLC1f-Cre transgenic mice.

To generate inducible muscle-specific TFEB transgenic animals, Tcfeb3XFlag cDNA was inserted after a CAGCAT cassette [chicken actin promoter (CAG) followed by chloramphenicol acetyltransferase (CAT) cDNA flanked by 2 loxP sites] and used to generate transgenic mice (Baylor College of Medicine transgenic core) (Settembre et al., 2011). Mice were crossed with transgenic mice expressing Cre-ER driven by human skeletal actin promoter (HSA). Tamoxifen-induced Cre LoxP recombination was activated by IP injection of tamoxifen-containing for 3 days. Muscles were collected 2 weeks after the tamoxifen treatment. Cre-negative littermates, also receiving tamoxifen treatment, were used as controls. Adult mice (3 to 5 months-old) of the same sex and age were used for each individual experiment.

All mice used were males and maintained in a C57BL/6 strain background. For all experiments involving TFEB-KO mice, the control mice were mice Tcfeb\(^{f/f}\) that did not carry the MLC1f-Cre transgene. Standard food and water were given ad libitum. Mice were maintained in a temperature- and humidity-controlled animal-care facility, with a 12 hr light/dark cycle and free access to water and food (Standard Diet, Mucedola, Italy). All experiments were performed on 3- to 5-month-old male (28–32g), mice of the same sex and weight for each individual experiment. In vivo TFEB overexpression experiments were performed by i.m. injection of a total dose of 10\(^{11}\) GC of AAV2/1 vector preparation. Muscles were removed 21 days after injection and frozen in liquid nitrogen for subsequent analyses. For fasting experiments, control animals were fed ad libitum; food pellets were removed from the cages of the fasted animals. All procedures were formerly approved by the Italian Public Health, Animal Health, Nutrition and Food Safety, Italian Ministry of Health, (D.M. N°75/2014-B).

**Western Blot Analysis and Antibodies.**

For western blot the following antibodies were used: anti-GAPDH (cat. sc-32233) anti-nNOS (cat. sc-648) from Santa Cruz Biotechnology; anti-NDUFA9 (cat.459100), anti-SDHA (cat. 459200), anti-COX5a (cat. 459120), from Invirotogen; anti-Gys (cat. 3886), anti-pGys (cat. 3891), anti-AMPK (cat.2532), anti-pAMPK (cat. 2531), anti-pACC (cat. 3661), anti-AKT (cat. 4691) from Cell Signaling; anti-GLUT1(cat. ab40084) from Abcam; anti-LC3B (cat. L7543), anti-p62 (cat. P0067) from Sigma Aldrich.

**Biochemical Analysis of Mitochondrial Respiratory Chain Complex.**

Complex I activity was measured using 50 μg mitochondrial protein in a reaction containing 250 mM succrose, 2 mM EDTA, 100 mM Tris–HCl pH 7.4, between 10 and 100 μM decyl-ubiquinone and 2 mM KCN at 30°C. Reactions were started by the addition of 50 μM NADH and oxidation followed at 340 nm for 2 minutes. Rotenone was used to block any rotenone insensitive activity.

Complex II activity was measured by incubating 40 μg of mitochondrial protein at 30°C in a reaction mix containing 50 mM KH2PO4, pH 7.4, 20 mM succinate, 2 μg/mL antimycin A, 2 μg/mL rotenone, 2 mM KCN and 50 μM 2,6-dichlorophenolindophenol (DCPIP). Reactions were initiated by adding 50μM DB to the reaction and measuring the change in absorbance at 600 nm for 2 min.

Complex III activity is measured by incubating 20 μg mitochondrial proteins at 30°C in a reaction mix containing 250 mM succrose, 50 mM Tris–HCl, 1 mM EDTA, 50 μM cytochrome c and 2 mM KCN. Decylubiquinol (DBH2) was used as an electron donor. The reaction was started by the addition of 50 μM DBH2 and the change in absorbance of cytochrome c at 550 nm was followed for 2 min. 5 μg of antimycin A were added to duplicate reactions to measure any antimycin A-insensitive activity. Complex IV activity was measured by adding 10 μg mitochondria to a mix containing 10 mM KH2PO4, pH 7.2 and between 5 and 50 μM ferrocytochrome c. The rate of oxidation of ferrocytochrome c was recorded for 2 min (extinction coefficient of 27.2 mM–1 cm–1). Citrate synthase activity was measured at 30°C by using 10 μg mitochondria to a
reaction mixture containing 125 mM Tris–HCl, 100 μM DTNB (5,5′-dithiobis(2-nitrobenzoic acid)) and 300 μM acetyl coenzyme A. The reaction is initiated by the addition of 500 μM oxaloacetate, and DTNB reduction at 412 nm measured for 2 min. The Mitochondrial respiratory chain activities were expressed as nmol/min/mg of protein.

For the quantitative determination of Adenosine 5′triphosphate (ATP) the muscle gastrocnemius samples stored in liquid nitrogen were homogenized in sterile water and analyzed according to the manufacturer’s instructions of ATP Bioluminescent Assay Kit (Sigma-Aldrich).

**Protein Carbonyls Detection.**

Carbonylation of muscle proteins was detected by using the OxyBlot Protein Oxidation Detection Kit from Millipore (Masiero et al., 2009). Spot detection and quantification was carried out using the UVP VisionWorksLS software.

**Acute Exercise, Training and Fatigue Experiments**

Mild exercise was performed by let mice run on the treadmill for 1 hour at 15 cm/sec speed for 4 days. Training was performed by increasing the speed of 5 cm/sec every week for a total of 7 weeks. The starting speed was 50% of the maximal speed (exhaustion test) that corresponded to 25 cm/sec and the final speed reached at the end of the training period was 55 cm/sec. To measure muscle endurance, soleus muscles were dissected under a stereomicroscope and mounted between a force transducer (KG Scientific Instruments, Heidelberg, Germany) and a micromanipulator-controlled shaft in a small chamber in which oxygenated Krebs solution was continuously circulated and temperature maintained at 25°C. The stimulation voltage was optimized, and the length of the soleus was increased until force development during tetanus was maximal. The responses to a single stimulus (twitches) or to trains of stimuli at various rates producing unfused or fused tetani were recorded. In order to induce muscle fatigue, muscles were stimulated for 500ms at 100Hz every 2 seconds (0.25 duty cycle). Induction of fatigue ex-vivo is known to be performed at a duty cycle between 0.1 and 0.5 (Allen et al., 2008).

**Mitochondrial Assays**

Mitochondria from mouse skeletal muscle were isolated as described (Frezza et al., 2007). The rate of mitochondrial oxygen consumption was measured at 30°C in an incubator chamber with a Clark/type O2 electrode filled with 2ml of incubation medium (125 mM KCl, 10 mM Pi, Tris, 20 mM Tris-HCl, 0.1 mM EGTA, ph 7.2). All measured were performed using mitochondria (0.2 mg mitochondrial protein/ml) incubated either with glutamate (5 mM)/ malate (2.5 mM) or /and succinate (5 mM) as substrates, in the presence (state 3) and in absence (state 4) of 100 mM ADP (Frezza et al., 2007).

**Autophagic flux quantification**

We monitored autophagic flux in fed and 24 h starving mice by using colchicine as previously described (Milan et al., 2015). Briefly muscle specific transgenic TFEB and TFEB-KO mice were treated with 0.4 mg/kg colchicine or vehicle by i.p. injection and starved. The treatment was repeated at 15 h prior to muscle harvesting.

**Morphological Analysis.**

For SDH activity, 8μm thick sections from frozen tissue were collected on coverslips. The reaction mix contained: 5 mM phosphate buffer pH 7.4, 5 mM EDTA, 1 mM KCN, 0.2 mM Phennazine methosulfate (PMS), 50 mM Succinic acid, 1.5 mM Nitro blue tetrazolium (NBT). NBT was the electron acceptor with PMS served as intermediate electron donor to NBT. Sections were incubated for 20 min at 37°C. For control sections, sodium malonate (10 mM) was added to the incubation medium. For Cytochrome-c Oxidase the sections were incubated 1 hr at 37°C. The reaction mix contained: 5 mM phosphate buffer pH 7.4, 0.1% 3,3-Diaminobenzidine (DAB), 0.1% Cytochrome c, 0.02% catalase. Histochemical analyses were performed as described (Sciacco and Bonilla, 1996). Cross-sectional area was measured using ImageJ software in at least 400 fibres and compared with the area of age-matched control. The fibre diameter was calculated as caliper width, perpendicular to the longest chord of each myofibre. The total myofibre number was calculated from entire muscle section based on assembled mosaic image (×20 magnification). (Fibre typing was determined by immunofluorescence using combinations of the following monoclonal antibodies: BA-D5 that recognizes type 1 MyHC isoform and SC-71 for type 2A MyHC isoform. Images were captured using a Leica DFC300-FX digital charge-coupled device camera by using Leica DC Viewer software, and morphometric analyses were made using the software ImageJ 1.47 version.
**Immunohistochemistry**

Frozen muscle serial sections (7 μm) were fixed in 4% paraformaldehyde in PBS for 10 min, washed twice in PBS (Sigma-Aldrich) for 5 min at room temperature (RT), and incubated in 0.03% H₂O₂ in Methanol for 10 min at RT. After washing in PBS, sections were incubated in blocking solution (10% normal goat serum, 5% BSA in PBS) for 1 h at RT. Anti-TFEB mAb (Bethyl Laboratories) was added separately to each serial section for 16 h at 4°C. Sections were washed twice in PBS, incubated with biotin-conjugated goat anti-rabbit IgG1, 5 μg/ml with 1% normal goat serum and 0.5% BSA in PBS) for 2 h at 4°C, washed twice, incubated with HRP-conjugated streptavidin (NovaRED substrate kit for peroxidase) (Vector Laboratories), following the manufacturer instructions, then rinsed in distilled water for 5 min, and counterstained with hematoxylin. Sections were prepared from independent muscle specimens from 3 mice for group; and 3–4 sections for each muscle were analyzed.

**Electron microscopy.**

Small pieces of muscle tissue were fixed in a mixture of 2% paraformaldehyde and 1% Glutaraldehyde prepared in 0.2 M Hepes. Then samples were post-fixed in a mixture of osmium tetroxide and potassium ferrocyanide, dehydrated in ethanol and propyleneoxide and embedded in epoxy resin as described previously (Polishchuk et al., 2014). Thin 65 nm sections were cut using a Leica EM UC7 ultramicrotome. EM images were acquired using a FEI Tecnai-12 electron microscope (FEI, Eindhoven, Netherlands) equipped with a VELETTA CCD digital camera (Soft Imaging Systems GmbH, Munster, Germany). Morphometric analysis of number and size of mitochondria was performed using iTEM software (Olympus SIS, Germany). Number of mitochondria was counted using the same magnification within 100 μm square field of view. For each experiment, between 154 and 196 individual mitochondrial diameters were measured from 3 mice per group.

**Tissue metabolites quantification**

Muscle free fatty acids were extracted as follows: briefly, pulverized muscle was homogenized in PBS, then extracted using chloroform/methanol (2:1), dried overnight and re-suspended in a solution of 60% butanol 40% Triton X-114/methanol (2:1). Measurements were normalized to protein content in the initial homogenate by DC protein assay (Bio-Rad). The quantitative determination of glycogen amount was performed according to the manufacture’s instruction of the Glycogen Colorimetric/Fluorometric Assay Kit (BioVision). For the quantitative determination of ketone bodies, the muscle gastrocnemius samples stored in liquid nitrogen were homogenized and analyzed according to the manufacturer’s instructions of β-hydroxybutyrate colorimetric assay kit (Vinci-Biochem). β-Hydroxybutyrate concentration is determined by a coupled enzyme reaction, which results in a colorimetric (450 nm) product, proportional to the β-Hydroxybutyrate present in muscle.

**Whole Body Indirect Calorimetry.**

Mice were individually housed in the chamber with a 12-h light/12-h dark cycle in an ambient temperature of 22–24°C. VO₂ and VCO₂ rates were determined under Oxymax system settings as follows: air flow, 0.6 l/min; sample flow, 0.5 l/min; settling time, 6 min; and measuring time, 3 min. The system was calibrated against a standard gas mixture to measure O₂ consumed (VO₂, ml/kg/h) and CO₂ generated (VCO₂, ml/kg/h). Energy expenditure (EE), oxygen consumption (V O₂) and carbon dioxide production (Vco₂) during running was determined as described by Shemesh (Shemesh et al., 2014). Briefly, the mice were acclimatized to a treadmill (Columbus Instruments) by running at 10 m/min for 15 min over three consecutive days. On the fourth day, groups of mice were run at 10 m/min, progressing to 15 m/min. Energy expenditure, oxygen consumption (V O₂) and carbon dioxide production (V CO₂) were recorded during running interval, and the total distance run to exhaustion was determined.

**In Vivo Assessment of Insulin Action and Glucose Metabolism.**

The 2-h EU clamp was conducted with a prime continuous infusion of human insulin (4 mill units/kg/min) and a variable infusion of 25% glucose to maintain glucose at <150 mg/dl. Insulin stimulated whole body glucose metabolism was estimated using a prime continuous infusion of [3-3H] glucose (10 μCi bolus, 0.1 μCi/min; PerkinElmer Life Sciences). To determine the rate of basal glucose turnover, [3-3H] glucose (0.05 μCi/min) was infused for 2 h (basal period) before starting the EU clamp, and a blood sample was taken at the end of this basal period. To assess insulin-stimulated tissue-specific glucose uptake, 2-deoxy-d-[1-14C] glucose (PerkinElmer Life
Sciences) was administered as a bolus (10 μCi) 75 min after the start of the clamp. Blood samples were taken at 80, 90, 100, 110, and 120 min after the start of the EU clamp. At the end of the EU clamp, different muscle groups, adipose tissue, and liver were rapidly dissected and frozen at -80°C for analysis. For the determination of plasma [3-3H]glucose and 2-deoxy-d-[1-14C]glucose concentrations, plasma was deproteinized with ZnSO4 and Ba(OH)2, dried to remove 3H2O, resuspended in water, and counted in scintillation fluid (Ultima Gold; Packard Instrument Co.). For the determination of tissue 2-deoxy-d-[1-14C]glucose (2-DG)-6-phosphate (2-DG-6-P) content, tissue samples were homogenized, and the supernatants were subjected to an ion-exchange column to separate 2-DG-6-P from 2-DG, as described previously. The radioactivity of 1H in tissue glycogen was determined by digesting tissue samples in KOH and precipitating glycogen with ethanol as previously described. Muscle glycogen synthesis was calculated as muscle [3H] glycogen content divided by the area under the plasma [3H] glucose-specific activity profile.

**Microarray hybridization.**
Total RNA (3 μg) was reverse transcribed to single-stranded cDNA with a special oligo (dT) 24 primer containing a T7 RNA promoter site, added 3' to the poly-T tract, prior to second strand synthesis (One Cycle cDNA Synthesis Kit by Affymetrix, Fremont, CA, USA). Biotinylated cRNAs were then generated, using the GeneChip IVT Labeling Kit (Affymetrix). Twenty micrograms of biotinylated cRNA was fragmented and 10 μg hybridized to the Affymetrix GeneChip Mouse 430A-2 microarrays for 16 hours at 45°C using an Affymetrix GeneChip Fluidics Station 450 according to the manufacturer's standard protocols.

For determine the changes in muscle transcriptome induced by TFEB overexpression, total RNA was extracted from muscle from three mice injected with an adenoviral vector that expresses mouse Tcfeb (AAV2.1-TFEB) and muscle from three mice injected with AAV2.1-GFP used as control. For the analysis of Tcfeb knock-out (KO) mice muscle-specific total RNA was extracted from the muscle of three TFEB-KO mice 3 month old as compared to three WT mice used as control.

**Microarray data processing.**
The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO)(Edgar et al., 2002) and are accessible through GEO Series accession number GSE62975 (TFEB overexpression muscle dataset) and GSE62976 (TFEB-KO muscle dataset). The two muscle studies (GSE62975 and GSE62976) are part of the SuperSeries GSE62980. The SuperSeries has been named: Expression data from mice after knockout or overexpression of Tcfeb in muscle. Low-level analysis to convert probe level data to gene level expression was performed using Robust Multiarray Average (RMA) implemented using the RMA function of the Bioconductor project (Gentleman et al., 2004a, b).

**Statistical analysis of differential gene expression.**
For each gene, a Bayesian t-test (Cyber-t) (Baldi and Long, 2001) was used on RMA normalized data to determine if there was a significant difference in expression between mice overexpressing TFEB (TFEB overexpression muscle mice) versus not-injected mice used as control and Tcfeb knock-down (KO) mice muscle-specific versus WT mice. P-value adjustment for multiple comparisons was done with the False Discovery Rate (FDR) of Benjamini-Hochberg (Klipper-Aurbach et al., 1995). The threshold for statistical significance chosen for both muscle microarray datasets analysis was FDR<0.05; a further filtering was performed for TFEB overexpression muscle dataset (GSE62975) by selection genes with an absolute Fold Change ≥1.5 for both increased (up-regulated genes) and decreased (down-regulated genes) expression levels.

**Microarray data analysis.**
Gene Ontology Enrichment Analysis (GOEA) (Dennis et al., 2003) was performed for each microarray dataset on the up-regulated and down-regulated gene lists, separately, by using the DAVID online tool (DAVID Bioinformatics Resources 6.7) restricting the output to all Biological Process terms (BP_ALL) and to all Cellular Compartments terms (CC_ALL). The threshold for statistical significance of GOEA was FDR≤10% and Enrichment Score ≥1.5. 73 out of 461 genes significantly inhibited in the TFEB-KO muscle dataset were mainly localized in the mitochondrion (GO:0005739) as shown in the Table S5. Promoter analysis to isolate potential TFEB binding sites (i.e. CLEAR motifs) was also performed on a region of 1kb up-stream the TSS of the human and mouse promoter sequences of the 73 Mitochondrial genes; 52 out of the total 73 Mitochondrial genes contain at least one CLEAR motif in the human or mouse promoter, or in both (Table S6, 73 Mitochondrial_DW_TFEB-KO_CLEAR). 38 out of 1512 genes significantly induced in the TFEB overexpression muscle dataset were localized in the
mitochondrial part (GO:0044429; refer to Table S4). In Figure 1A the Lipid and Glucose Metabolism related terms induced by TFEB overexpression and inhibited by TFEB-KO were selected from BPALL_UP in TFEB overexpression and from BPALL_DOWN in TFEB-KO, respectively. For each term the genes significantly induced by TFEB overexpression and inhibited by TFEB-KO are reported in table Table S3 Lipid and Glucose Metabolism.

**Accession codes:**
Primary accessions Gene Expression Omnibus
The two muscle studies (GSE62975 and GSE62976) are part of the SuperSeries GSE62980. The SuperSeries has been named: Expression data from mice after knockout or overexpression of Tcfeb in muscle.

**GSE62975:** Expression data from injected mice overexpressing Tcfeb specifically in muscle

**GSE62976:** Expression data from Tcfeb KO mice specifically in muscle
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