TFE3 regulates whole-body energy metabolism in cooperation with TFEB

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Abstract

TFE3 and TFEB are members of the MiT family of HLH–leucine zipper transcription factors. Recent studies demonstrated that they bind overlapping sets of promoters and are post-transcriptionally regulated through a similar mechanism. However, while Tcfe3 knockout (KO) mice die during early embryonic development, no apparent phenotype was reported in Tcfbe3 KO mice. Thus raising the need to characterize the physiological role of TFE3 and elucidate its relationship with TFEB. TFE3 deficiency resulted in altered mitochondrial morphology and function both in vitro and in vivo due to compromised mitochondrial dynamics. In addition, Tfe3 KO mice showed significant abnormalities in energy balance and alterations in systemic glucose and lipid metabolism, resulting in enhanced diet-induced obesity and diabetes. Conversely, viral-mediated TFE3 overexpression improved the metabolic abnormalities induced by high-fat diet (HFD). Both TFEB overexpression in Tfe3 KO mice and TFE3 overexpression in Tcfbe3 liver-specific KO mice (Tcfeb LiKO) rescued HFD-induced obesity, indicating that TFEB can compensate for TFE3 deficiency and vice versa. Analysis of Tcfeb LiKO/Tfe3 double KO mice demonstrated that depletion of both TFE3 and TFEB results in additive effects with an exacerbation of the hepatic phenotype. These data indicate that TFE3 and TFEB play a cooperative, rather than redundant, role in the control of the adaptive response of whole-body metabolism to environmental cues such as diet and physical exercise.

Keywords lipid metabolism; mitochondrial dynamics; physical exercise; TFE3; TFEB

Introduction

Complex organisms have developed sophisticated and efficient mechanisms to adapt their energy metabolism to environmental cues. These mechanisms typically involve both protein post-translational modifications and protein–protein interactions for acute responses, and transcriptional regulation for more chronic sustained responses. The transcription factor EB (TFEB) plays an important role in the control of cell and organismal homeostasis (Settembre et al, 2013b; Napolitano & Ballabio, 2016). TFEB responds to cellular nutrient levels and regulates lysosomal biogenesis and autophagy (Sardiello et al, 2009; Settembre et al, 2011). Under nutrient-rich conditions, TFEB resides in the cytoplasm and translocates to the nucleus in response to starvation and lysosomal stress (Settembre et al, 2011). In the liver, fasting activates TFEB, which promotes lipophagy and lipid catabolism via induced expression of the transcriptional co-activator Pgc1α and nuclear receptor Pparα (Settembre et al, 2013a).

TFEB belongs to the MiT family of helix-loop-helix leucine zipper transcription factors, together with TFE3, MITF and TFEC (Sten Grimsson et al, 2004). Recently, it has been demonstrated that TFE3 and TFEB regulate overlapping sets of genes and their overexpression has similar effects (Martina et al, 2014). Subcellular localization of both TFE3 and TFEB is controlled by their phospho-tyrosine status on particular serine residues (Settembre et al, 2011, 2012; Rocznia-Ferguson et al, 2012; Martina et al, 2014; Medina et al, 2015; Li et al, 2016). A variety of stimuli are able to induce TFE3 and TFEB cytoplasm-to-nucleus translocation (Napolitano & Ballabio, 2016). Recent studies demonstrated that pathogen infections promote TFE3 and TFEB nuclear translocation, thereby inducing the expression of several cytokines and chemokines (Visvikis et al, 2014; Najibi et al, 2016; Pastore et al, 2016).

In spite of the similarities between TFE3 and TFEB, it is still unclear whether these transcription factors have similar, cooperative, complementary or redundant roles in mammalian physiology. In particular, the characterization of the physiological role of TFE3 has been hampered by the lack of an apparent phenotype in Tfe3

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KO mice (Steingrimsson et al., 2002), whereas Tfe3 KO mice die during embryonic development (Steingrimsson et al., 1998).

In this study, by analysing mice lacking Tfe3, we discovered that TFE3 plays a crucial role in the regulation of lipid and glucose metabolism and is necessary for proper mitochondrial dynamics and function in both liver and muscle. Moreover, epistatic analysis revealed that TFE3 and TFEB cooperate in the metabolic adaptation to physiological stressors in multiple organs.

**Results**

**Tfe3 KO mice have an altered energy balance and glucose homeostasis**

To investigate the physiological role of TFE3, we performed a thorough phenotypic and metabolic analysis of previously generated Tfe3 KO mice (Steingrimsson et al., 2002). No main differences were observed in body weight between wild-type (WT) and Tfe3 KO mice fed a chow diet (Fig 1A). However, magnetic resonance imaging (MRI) revealed a 50% increase in fat mass and a significant reduction in lean mass (Fig 1B) in Tfe3 KO mice compared to controls. These alterations could not be attributed to differences in food intake, as food consumption was similar between the two genotypes (Fig 1C). Tfe3 KO mice displayed enlarged fat depots and increased epididymal (eWAT) and inguinal (iWAT) fat weight with significant reduction in liver weight (Fig 1D). Consistent with increased adipose tissue weight, plasma leptin levels were elevated, while adiponectin levels demonstrated a trend towards reduction but did not reach statistical significance (Fig 1E). Comprehensive laboratory animal monitoring system (CLAMS) studies, used to measure metabolic activities, showed that during the light phase, WT and Tfe3 KO animals had a similarly low respiration exchange ratio (RER, \( V_{CO2} / V_{O2} \)), which likely reflects the shift from carbohydrate to fatty acid consumption and the changes in activity and food intake (Fig 1F and G). Tfe3 KO mice did not show significant differences in \( O_2 \) consumption (Fig EV1A and B) or \( CO_2 \) production (Fig EV1C and D) during the light and dark phases compared to WT controls. Notably, Tfe3 KO mice showed reduced energy expenditure (Fig 1H and I, and Appendix Fig S1A and B) that could explain their increased adiposity.

To test whether Tfe3 KO mice have abnormalities in glucose homeostasis, we analysed their blood glucose levels. No differences were observed among the genotypes under basal feeding conditions; however, blood glucose levels of 24-h-fasted Tfe3 KO mice were significantly lower than those of fasted controls (Fig 2A). Tfe3 KO mice also showed increased insulin (Fig 2B), and reduced glucagon (Fig 2C) levels in plasma. We then further examined glucose homeostasis by performing glucose tolerance test (GTT) and insulin tolerance test (ITT). While no differences were observed in ITT between the genotypes (Fig 2D), Tfe3 KO mice showed glucose intolerance (Fig 2E), which was associated with reduced insulin secretion 15 min after glucose injection (Fig 2F). Tfe3 KO mice also displayed altered hepatic gluconeogenesis during a PTT test (Fig 2G) and the expression of Pepck and G6Pc, key players in gluconeogenesis, was reduced in the Tfe3 KO livers (Fig 2H), indicating insufficient gluconeogenesis as a contributing factor to the reduced fasting glucose. Periodic acid–Schiff (PAS) staining revealed a reduction in glycogen stores in liver (Fig EV1E and F) as well as in muscle (Fig EV1G) of Tfe3 KO mice compared to their WT littermates. During exercise, blood glucose levels and muscle glycogen stores were similarly depleted in WT and Tfe3 KO mice (Fig 2I and J). However, whereas WT animals were able to restore their blood glucose and muscle glycogen stores following a 2-h recovery period, Tfe3 KO animals trailed behind in their blood glucose recovery and completely failed to replete muscle glycogen stores (Fig 2I and J). Moreover, we observed a higher increase in blood lactate levels in Tfe3 KO mice following exercise (Fig 2K), suggesting that they utilize anaerobic glycolysis as a source of energy. Failure to replete glycogen stores in muscle following exercise can occur as a result of impaired muscle glucose uptake or glycogen synthesis. Indeed, Gok3 expression was significantly reduced in Tfe3 KO muscle (Fig 2L), suggesting that reduced glycogen synthesis may occur. Consistent with a role of TFE3 in glycogen homeostasis, viral-mediated TFE3 overexpression in WT mice injected systemically using an helper-dependent adenovirus (HDAd) expressing the human TFE3 gene under the control of a promoter mainly expressed in the liver (HDAd-PEPCK-hTFE3) or intramuscularly (i.m.) using an adenov-associated virus (AAV) expressing the human TFE3 gene under the control of a ubiquitous promoter (AAV2.1-CMV-hTFE3) increased glycogen stores in both liver and muscle (Fig EV1H).

These data indicate that TFE3 plays a key role in regulating whole-body glucose and glycogen homeostasis.

**Abnormalities in lipid metabolism in Tfe3 KO mice**

Since Tfe3 KO mice show an increased adiposity (Fig 1B and D), we evaluated whether lipid homeostasis was also altered. We investigated the ability of Tfe3 KO mice to utilize fat in response to starvation. Under basal feeding conditions, livers from Tfe3 KO mice displayed normal morphology and gross appearance with no apparent histological abnormalities (Figs 1D and EV2A). Conversely, following 24-h starvation, Tfe3 KO mice exhibited marked hepatic steatosis as determined by gross inspection, H&E and Oil Red O staining (Fig EV2A and B). Electron microscopy (EM) analysis (Fig EV2C) and the direct quantification of total lipid content in the liver (Fig EV2D) confirmed an increased accumulation of lipids in Tfe3 KO livers upon fasting. Moreover, several metabolic parameters (ALT, AST, CK, LDH) were significantly increased in Tfe3 KO mice in response to starvation (Appendix Table S1) suggesting liver stress. A reduced expression of genes involved in lipid metabolism (e.g. Cldn6, Cyp7a1, Fgf21, Cpt1a, Pgc1a, ApoA4, Cyp17a1, Cyp4a10, Cyp4a14) was detected in 24-h-fasted Tfe3 KO livers and primary hepatocytes as compared to fasted WT (Fig EV2E and F). Moreover, a reduction in the expression of genes involved in lipogenesis (e.g. Fasn and Sreb1c) was detected in Tfe3 KO mice in the fed state (Fig EV2E). As expected, opposite effects were observed in WT mice systemically injected with the HDAd-PEPCK-hTFE3, which showed increased expression of genes involved in lipid metabolism in the liver when compared to controls (Fig EV2G).

To further evaluate lipid homeostasis, we fed Tfe3 KO mice and controls with a high-fat diet (HFD) for 18 weeks. HFD-fed Tfe3 KO mice gained significantly more weight (Fig 3A and B) and showed a pale liver and increased adipose mass (Fig 3A) compared to WT mice. Indeed, MRI analysis revealed a further increase in fat mass with no reduction in lean mass in Tfe3 KO mice compared to WT
This was not due to increased food intake, which did not significantly differ between the genotypes (Fig EV3A). Tfe3 KO mice showed hyperleptinaemia, hypoadiponectinaemia, hyperinsulinaemia and hypercholesterolaemia compared to controls (Fig EV3B). Although FFA levels showed a trend for increase, they were not significantly different (Fig EV3B). Indirect calorimetry showed a significant increase in RER (VCO2/VO2) values indicating a preferential utilization of carbohydrates and reduced lipid catabolism in Tfe3 KO mice fed with a HFD (Fig 3D and E), which explains the obesity-prone phenotype. They also showed increased O2 consumption (Fig EV3C and D) and CO2 production (Fig EV3E and F), but reduced energy expenditure per body weight (Fig 3F and G, and Appendix Fig S2A and B), which accounts for the impaired energy balance in Tfe3 KO mice.

Excessive adiposity and obesity are linked to insulin resistance (Kahn & Flier, 2000). Consistent with these findings, Tfe3 KO mice showed enhanced glucose intolerance and insensitivity, as well as insulin resistance, as indicated by glucose and insulin levels during GTT (Fig EV3G and H) and ITT (Fig EV3I) after 1 month of HFD.
Muscle glucose uptake was also impaired in HFD-fed Tfe3 KO mice (Fig EV3J). In line with the severe obesity, in vivo-stimulated lipolysis was impaired in Tfe3 KO mice (Fig EV3K). Histological analysis performed after 18 weeks of HFD revealed a marked vacuolar degeneration and fat accumulation in the liver of Tfe3 KO mice (Fig 3H and I), as well as enlarged lipid droplets in both eWAT and
Figure 3. TFE3 regulates lipid catabolism.

A Body size and tissues’ gross appearance from WT and Tfe3 KO mice after 1 month of high-fat diet (HFD).

B Body weight gained over time in control and Tfe3 KO mice fed a HFD for 18 weeks (n = 6 per group). Data are presented as mean ± SEM. ANOVA test followed by post hoc Bonferroni test: ***P < 0.001.

C Visceral fat and lean mass from mice with the indicated genotypes after 1 month on HFD (n = 5 per group). Data are presented as mean ± SEM. Student’s two-tailed t-test: **P = 0.0041.

D Respiratory exchange ratio (RER; VCO2/VO2) in WT (black line) and Tfe3 KO (red line) mice after 1 month of HFD (n = 5 per group). Grey areas indicate dark periods (6 PM to 6 AM). Data are presented as mean ± SEM.

E Bar graph represents RER average values during day and night (n = 5 per group). Data are presented as mean ± SEM. Student’s two-tailed t-test: day *P = 0.0149; night *P = 0.0270.

F Energy expenditure (EE) normalized for body weight in WT (black line) and Tfe3 KO mice (red line) after 1 month of HFD (n = 5 per group). Grey areas indicate dark periods (6 PM to 6 AM). Data are presented as mean ± SEM.

G Bar graph represents average EE values during day and night (n = 5 per group). Data are presented as mean ± SEM. Student’s two-tailed t-test: day *P = 0.0485; night *P = 0.0227.

H Liver triglycerides (TG) levels in WT and Tfe3 KO mice after 18 weeks of HFD (n = 5 per group). Dashed line indicates WT fed a chow diet. Data are presented as mean ± SEM. Student’s two-tailed t-test: ****P < 0.0001.

I, J H&E, Oil Red O staining and EM images of liver sections (I) and H&E staining of eWAT, BAT and muscle sections (J) from Tfe3 KO and control mice fed a HFD for 18 weeks (scale bars liver, eWAT, BAT: 50 μm; scale bars muscle: 20 μm).
brown adipose tissue (BAT; Fig 3J). Importantly, Tfe3 KO mice showed down-regulation of genes involved in thermogenesis, including Ucp1 and Ucp3, and oxidative genes such as Ppara in the BAT (Appendix Fig S3A and B), which account, at least partially, for their reduced energy expenditure and diet-induced obesity. A higher content of lipid droplets was also observed in skeletal muscle after HFD (Fig 3J).

Thus, the absence of TFE3 in mice results in enhanced diet-induced obesity and diabetes, indicating that TFE3 plays an important role in lipid metabolism, metabolic homeostasis and in the adaptive response to dietary lipids.

TFE3 controls the response to physical exercise

Exercise elicits several beneficial effects by acting on mitochondrial content/function, fatty acid oxidation and glucose homeostasis (Holloszy & Coyle, 1984; Holloszy et al., 1998; Hawley, 2002). Physical activity is also important to counteract disease progression in diabetes, obesity and metabolic syndrome. Tfe3 KO mice performed significantly worse compared to their WT littersmates in an exhaustive endurance test (Fig 4A), indicating impaired endurance capacity. In order to test whether TFE3 is activated by exercise, we electroporated a CMV-TFE3-GFP plasmid in the tibialis anterior (TA) muscle of WT mice and performed an acute bout of exercise 8 days later. As evident by increased nuclear GFP signal in muscle and subcellular fractionation analysis in liver, TFE3, as well as TFEB, translocate to the nucleus in response to exercise (Fig 4B–D). We then subjected WT and Tfe3 KO mice fed with a HFD to 8 weeks of exercise training. While WT mice greatly benefited from chronic exercise training, as evident by the reduced body (Fig 4E and F), liver (Fig 4G) and WAT weight gain (Fig 4H) and improved glucose tolerance (Fig 4I), Tfe3 KO mice did not show the same amelioration in adiposity (Fig 4F and H) and glucose tolerance (Fig 4I) and displayed a reduced improvement in body weight (Fig 4F) and endurance capacity compared to the WT mice (Fig 4J).

These findings implicate TFE3 in mediating exercise-induced metabolic adaptations.

TFE3 regulates mitochondrial dynamics and function

Electron microscopy (EM) analysis of Tfe3 KO liver and muscle revealed an unexpected and pronounced accumulation of morphologically abnormal mitochondria (Fig 5A and B). In liver, there was no difference detected in mitochondrial number, but a significant increase in size was observed (Fig 5C), while mitochondrial number and size were both increased in muscle (Fig 5D). These data suggest that an alteration in mitochondrial dynamics may be at play.

During starvation, mitochondria elongate in WT primary hepatocytes as demonstrated by a network of highly interconnected organelles (Fig 5E), as previously reported (Gomes et al., 2011). In contrast, Tfe3 KO hepatocytes show round, bigger mitochondria, which remain enlarged but punctate regardless of nutritional status (Fig 5E). Indeed, the expression of genes involved in mitochondrial dynamics (e.g. Fis1, Drp1 and Mfn1) was reduced in Tfe3 KO liver (Fig 5F). In contrast, overexpression of TFE3 in liver induced the expression of genes involved in mitochondrial dynamics (Fis1, Drp1, Opa1, Mfn1 and Mfn2; Fig 5F). To understand whether TFE3 directly regulates mitochondrial gene expression, we analysed published chromatin immunoprecipitation sequencing (ChIP-seq) data of TFE3 in mouse ES cells (Betschinger et al., 2013). Gene ontology analysis identified 136 mitochondria-related genes targeted by TFE3. Analysis of the mitochondrial biological processes revealed that the fission pathway was highly enriched (Appendix Table S2). Indeed, Fis1, a mitochondrial fission receptor, was among the most highly enriched genes. Promoter analysis revealed two E-boxes in the promoter of Fis1 close to the TSS. ChIP-qPCR experiments in TFE3-overexpressing and KO livers confirmed that TFE3 binds directly to the Fis1 promoter, thus directly regulating its expression (Fig 5G).

We then tested whether the abnormalities in mitochondrial dynamics observed in Tfe3 KO mice were associated with defective mitochondrial biogenesis and function. We investigated the integrity of the electron transport chain (ETC), which is composed of sequentially acting electron carriers consisting of integral membrane proteins with prosthetic groups. Protein levels of complexes IV and V, as indicated by cytochrome c oxidase subunit 1 (mt-Co1) and ATP synthase, H⁺ transporting, mitochondrial F1 complex, alpha subunit 1 (Atp5a1), respectively, were not significantly changed in liver (Fig 6A and B) of Tfe3 KO mice. In contrast, complexes I, II and III, NADH:ubiquinone oxidoreductase subunit B8 (Ndub8), succinate dehydrogenase complex, subunit B (Sdhb) and ubiquinol-cytochrome c reductase core protein 2 (Uqrc2), were significantly down-regulated (Fig 6A and B) in Tfe3 KO livers. Consistently, TFE3 overexpression in the liver results in significant increase in complex I, II and III protein levels (Fig 6A and B).

Mitochondria are the main source of reactive oxygen species (ROS) in the cell and reduced complex I activity promotes mitochondrial ROS production (Verkaart et al., 2007). In line with the observed reduction in complex I activity, oxidative stress, as revealed by protein carbonylation, was significantly higher in Tfe3 KO livers compared to controls in line with their reduced complex I activity (Appendix Fig S4A). To determine whether mitochondria were responsible for the oxidative stress detected in Tfe3 KO mice, we transfected mouse embryonic fibroblasts from WT and Tfe3 KO mice with a ratio metric mitochondrial targeted ROS sensor. Confocal microscopy analysis demonstrated increased ROS levels in Tfe3 KO MEFs compared to controls (Appendix Fig S4B).

To evaluate mitochondrial function, we assessed mitochondrial membrane potential with TMRM in TFE3-depleted primary hepatocytes, HeLa and MEFs. Addition of oligomycin, an ATP-synthase inhibitor, caused a more robust reduction in membrane potential in Tfe3-depleted cells compared to control cells, while the protonophore carbonylcyanide-p-trifluoromethoxyphenyl hydrazine (FCCP), which was used as a positive control, completely depolarized both WT and Tfe3 KO cells (Fig 6C and D, and Appendix Fig S4C and D), indicating that mitochondria of Tfe3-depleted cells are compromised and require the reverse activity of ATP synthase to maintain their membrane potential. We then measured the respiratory capacity of primary hepatocytes lacking Tfe3 using the Seahorse system. Tfe3 KO hepatocytes showed a significant reduction in oxygen consumption rate (OCR) and markedly reduced spare respiratory capacity (SRC; Fig 6E). Moreover, basal oxygen consumption rate (OCR) and the uncoupled respiration were reduced, while coupling efficiency was unchanged (Fig 6F) in Tfe3 KO hepatocytes compared to controls, indicating a reduction in oxidative
Figure 4. TFE3 is required for exercise-induced metabolic benefits.

A Distance run to exhaustion by WT and Tfe3 KO mice fed a chow diet on an exhaustive endurance exercise test (n = 6 per group). Data are presented as mean ± SEM. Student’s two-tailed t-test: *P = 0.0333.

B Exercise-mediated nuclear translocation of TFE3 in tibialis anterior (TA) muscle from WT mice electroporated with a TFE3-GFP plasmid (scale bars: 50 μm). Arrows indicate GFP nuclear localization upon exercise.

C, D Representative immunoblots of enriched nuclear and cytosolic cellular subfraction from livers of WT exercised (Ex) and exercised mice that were allowed to recover for 2 h (Ex+R) (C) and relative quantification (D) (n = 4). Data are presented as mean ± SEM. Student’s two-tailed t-test: ***P = 0.0007, **P = 0.0017.

E, F Gross appearance (E) and body weight (F) of WT and Tfe3 KO mice fed a HFD with and without chronic exercise training (Ex) (n = 6 per group) at the end of the experiment. Data are presented as mean ± SEM. ANOVA test followed by post hoc Bonferroni test: ***P < 0.001, ****P < 0.0001.

C, H Liver (G) and WAT (H) weight of WT (n = 6) and Tfe3 KO (n = 4) mice fed a HFD with and without exercise training (Ex). Data are presented as mean ± SEM. Student’s two-tailed t-test: ***P < 0.001, *P = 0.0112.

I Glucose tolerance test in WT and Tfe3 KO mice fed a HFD with and without chronic exercise training (n = 3 per group). Data are presented as mean ± SEM. ANOVA test followed by post hoc Bonferroni test: ***P < 0.001.

J Distance run to exhaustion by exercise-trained and sedentary WT and Tfe3 KO mice fed a HFD on an endurance exercise test (n = 5 per group). Data are presented as mean ± SEM. Student’s two-tailed t-test: ***P = 0.0008, ****P < 0.0003, *P = 0.0250.

Source data are available online for this figure.
Figure 5. TFE3 controls mitochondrial dynamics.

A, B Electron microscopy analysis of liver (A) and skeletal muscle (B) from WT and Tfe3 KO mice fed a chow diet or 24-h-fasted mice showing mitochondrial morphology.

C, D Morphometrical analysis of liver (C) and skeletal muscle (D) of fed and 24-h-fasted Tfe3 KO mice revealed higher number and increased size of mitochondria compared to control mice. Quantification has been performed as reported in the Materials and Methods section. Data are presented as mean ± SEM. Student’s two-tailed t-test: liver *** P = 0.0001, ** P = 0.01 WT fed versus Tfe3 KO fed; ** P = 0.01 WT fasted versus Tfe3 KO fasted. Muscle number *** P = 0.0005, Tfe3 KO fed versus Tfe3 KO fasted * P = 0.05; WT fed versus Tfe3 KO fed * P = 0.019; WT fasted versus Tfe3 KO fasted * P = 0.0175. Muscle size **** P = 1.45E-05, ** P = 0.0066.

E Immunofluorescence analysis for TOM20 in primary hepatocytes isolated from WT and Tfe3 KO mice in control media or HBSS for 1 h (scale bars: 10 μm).

F Expression analysis of the genes involved in mitochondrial dynamics in liver from WT, Tfe3 KO and WT mice injected with an HDAd-PEPCK-hTFE3 virus resulting in liver-specific TFE3 expression (n = 4 per group). Data are presented as mean ± SEM. Student’s two-tailed t-test: Fis1 ** P = 0.0019, **** P < 0.0001, Drp1 ** P = 0.0071, * P = 0.0261; Opa1 ** P = 0.0029, Mfn1 ** P = 0.0076, *** P = 0.0006, Mfn2 * P = 0.0393.

G ChIP analysis of Fis1 promoter in liver extracts from WT, Tfe3 KO or TFE3-overexpressing mice and relative controls. Data are presented as mean ± SEM of two independent experiments. Student’s two-tailed t-test: **** P < 0.0001, * P = 0.0158.
Figure 6. TFE3 regulates mitochondrial function.

A, B OXPHOS protein levels in liver from WT, Tfe3 KO and TFE3-overexpressing mice fed a chow diet (A) and relative protein quantification (B) (n = 3 per group). Data are presented as mean ± SEM. Student’s two-tailed t-test: CIII Tfe3 KO ***P = 0.0003, TFE3OE ***P = 0.0002; CII Tfe3 KO **P = 0.0036, TFE3OE ***P = 0.0004; CI Tfe3 KO **P = 0.0088, TFE3OE **P = 0.0049.

C Mitochondrial membrane potential in primary hepatocytes isolated from WT and Tfe3 KO mice (n = 5 WT and n = 8 Tfe3 KO). TMRM signal was quantified as percentage of initial value. Where indicated, oligomycin (olm) or protonophore carbonylcyanide-p-trifluoromethoxyphenyl hydrazone (FCCP) was added. Data are presented as mean ± SEM.

D Representative images of TMRM signal at the indicated time points after olm addition (scale bars: 100 μm).

E Basal respiration, Uncoupled respiration, Coupling efficiency

F The basal OCR, uncoupled respiration (proton leak) and uncoupling efficiency (ATP turnover/basal OCR) calculated based on data in (E). Data are presented as mean ± SEM. Student’s two-tailed t-test: ****P < 0.0001; ***P = 0.0003.

G Coupling assay in mitochondria isolated from WT and Tfe3 KO livers (n = 4). Where indicated, ADP, olm, FCCP or antimycin A (AA) was added. Data are presented as mean ± SEM.

H OCR basal, at state 3 and state 3u determined based on data in (G). Data are presented as mean ± SEM. Student’s two-tailed t-test: *P = 0.0309, ****P < 0.0001.

Source data are available online for this figure.
phosphorylation. In addition, mitochondria isolated from Tfe3 KO livers showed a reduction in OCR in all stages (basal, state 3 and state 3u) compared to those from WT livers (Fig 6G and H). Collectively, these results implicate TFE3 in modulating mitochondrial function.

TFE3 cooperates with TFEB in the control of energy metabolism

To investigate whether TFE3 is sufficient to mitigate HFD-induced obesity and diabetes, we overexpressed TFE3 in the liver of WT mice by intravenous injection of the virus expressing the human TFE3 under the control of a promoter mainly expressed in the liver (HDAd-PEPCK-TFE3) and fed them with a HFD for 12 weeks. HFD feeding resulted in increased body weight in control mice (Fig 7A and B), while both early (HDAd-PEPCK-TFE3 injection prior to HFD administration) and late (HDAd-PEPCK-TFE3 injection 8 weeks into HFD) TFE3 overexpression in the liver resulted in reduced body and liver weights (Fig 7A–C) compared to control mice. GTT analysis following 4 weeks of HFD showed reduced blood glucose and improved glucose tolerance in TFE3-injected mice (Fig 7D). Moreover, mice overexpressing TFE3 exhibited a reduced number of lipid droplets and reduced hepatosteatosis as evident from liver sections stained with Oil Red O (Fig 7E). Furthermore, several metabolic parameters (ALT, AST, LDH) were reduced, indicating amelioration of liver stress (Appendix Table S3).

We previously demonstrated that Tcfеб liver-specific KO mice have abnormalities in lipid metabolism, which are significantly enhanced by HFD (Settembre et al., 2013a). To test whether TFE3 is sufficient to rescue the phenotype of Tcfеб-depleted mice, we systemically injected Tcfеб liver-specific KO (Tcfеб LiKO) mice...
Figure 8. TFE3 and TFEB cooperate in the control of energy metabolism.

A Cross appearance of WT, Tcfeb LIKO, Tfe3 KO and Tcfeb LIKO, Tfe3 KO (DKO) mice, liver and WAT after 12 weeks of HFD.
B Body weight from mice of the indicated genotypes (n = 10 per group). Data are presented as mean ± SEM. ANOVA test followed by post hoc Bonferroni test: **P < 0.01.
C Liver weight from mice of the indicated genotypes (n = 12 per group). Data are presented as mean ± SEM. Student’s two-tailed t-test: WT versus Tfe3 KO *P = 0.05; WT versus DKO ****P < 0.0001; Tcfeb LIKO versus DKO ***P = 0.0005; Tfe3 KO versus DKO *P = 0.0138.
D H&E (upper panel) and Oil Red O staining (lower panel) of liver sections of the indicated genotypes 12 weeks after the HFD (scale bars: 50 μm).
E Pyruvate tolerance test (PTT) of mice at the indicated genotypes following 1 month of HFD (n = 3 per group). Data are presented as mean ± SEM. ANOVA test followed by post hoc Bonferroni test: **P < 0.01.
treated with HFD with the HDAd-PEPCK-TFE3 virus. Remarkably, viral-mediated TFE3 overexpression in these mice completely compensated for the lack of TFEB and fully rescued the obese phenotype (Fig 7A), liver and body weight (Fig 7F and G) and the accumulation of lipid droplets (Fig 7H). Similarly, TFEB overexpression in the liver of Tfe3 KO mice treated with HFD also reduced weight gain and the accumulation of lipid droplets (Fig EV4A–C), indicating that the two family members, when overexpressed, are interchangeable in this scenario. In order to understand whether they are cooperative or redundant, we generated double KO (DKO) mice that lack both TFEB and TFE3 in the liver (Tcfeb liver KO; Tfe3 full KO). When fed with a HFD for 12 weeks, DKO mice showed an exacerbated increase in body size (Fig 8A and B), liver weight (Fig 8C) and lipid accumulation compared to the either of the single KO genotypes (Fig 8D). Moreover, PTT analysis showed that the depletion of both TFE3 and TFEB results in an additive reduction in glucose production (Fig 8E).

These results demonstrate that both TFE3 and TFEB are required for the regulation of lipid and glucose homeostasis and that the combined depletion of both transcription factors results in additive alterations in liver metabolism, indicating that they are cooperative rather than redundant.

**Discussion**

Previous studies using TFE3 transgenic mice showed that TFE3 is capable of activating genes involved in energy metabolism processes including glycolysis and insulin signalling (Nakagawa et al, 2006; Fujimoto et al, 2013; Kim et al, 2013; Xiong et al, 2016). However, as these studies were limited to overexpression, the physiological role of TFE3 has remained elusive. In the present study, we demonstrate that the absence of TFE3 causes systemic alterations in lipid and glucose metabolism. TFE3 depletion enhances diet-induced obesity and diabetes, while its overexpression has the opposite effects. Interestingly, we found that TFE3, like TFEB (Medina et al, 2015), translocates to the nucleus during exercise, suggesting its involvement in exercise-induced adaptive response. Consistently, we observed that TFE3 depletion results in diminished endurance and abolished exercise-induced metabolic benefits. Indeed, 8 weeks of exercise training failed to improve obesity, endurance capacity and glucose homeostasis in Tfe3 KO mice. This is likely due to the role of TFE3 in glucose homeostasis as Tfe3 KO mice failed to recover blood glucose and muscle glycogen following exercise.

We have previously reported that liver-specific loss of TFEB exacerbates diet-induced obesity (Settembre et al, 2013a). Here we show that TFE3 overexpression rescues the obese phenotype and the glucose intolerance in WT as well as Tcfeb LiKO mice fed HFD. TFE3 overexpression increases the expression of genes involved in lipid metabolism and reduces hepatic lipid accumulation. Strikingly, TFEB overexpression is also able to rescue obesity in Tfe3 KO mice. These results suggest that TFE3 and TFEB have partially redundant roles in cellular metabolism. Given their extensive amino acid sequence similarities and overlapping profiles, it is possible that, in steady-state conditions, TFEB and TFE3 are partially redundant in several tissues and that TFE3 may compensate for TFEB deficiency and vice versa. In support of this idea, TFE3 and TFEB have been shown to be mutually redundant transcriptional activators of genes encoding E-cadherin (Huan et al, 2005), CD40 ligand (Huan et al, 2006), PGC1α (Settembre et al, 2013a; Salma et al, 2015) and ATF4 (Martina et al, 2016), among others. However, in specific physiological contexts, either TFE3 or TFEB may play a more prominent role, a situation similar to that proposed for the differential regulation of
various NFAT subunits (Harris et al., 2006). This could explain the strikingly different effects observed between Tcfeb KO mice, which die during embryonic development, and Tfe3 KO mice which are viable. However, under certain conditions that require robust adaptations to environmental cues, such as during starvation, high-fat diet and physical exercise, both TFE3 and TFEB are required and their role becomes cooperative. More studies using conditional KO mice in different tissues and in different conditions are needed to completely decipher the redundant and cooperative roles of these two transcription factors.

Mitochondrial quality control is vital for maintaining cellular and organismal health. Indeed, mitochondrial dysfunction has been implicated in the pathogenesis of several metabolic diseases such as type 2 diabetes, obesity, cardiovascular disease and metabolic syndrome (Petersen et al., 2003). Mitochondrial fusion and fission, collectively referred to as mitochondrial dynamics, play an important role in maintaining mitochondrial vitality and thus cellular health (Gomes et al., 2011). How nutrients and the cellular metabolic state regulate mitochondrial dynamics is an area of active investigation. Here we show that Tfe3 null mice, previously described to be indistinguishable from their WT littermates, have severe mitochondrial abnormalities. Tfe3 deficiency results in accumulation of enlarged and dysfunctional mitochondria with impaired respiration leading to compromised lipid catabolism and exercise intolerance. Tfe3 null mice also show a reduced expression of genes involved in mitochondrial dynamics and oxidative metabolism, while TFE3 overexpression has the opposite effect.

In summary, our study reveals a central role for TFE3 in the regulation of glucose homeostasis, lipid metabolism and mitochondrial dynamics (Fig 9) and underlines the importance of this transcription factor in the metabolic response to environmental cues and pathological conditions.

Materials and Methods

Mouse experiments

Tfe3 KO mice were previously described (Steingrimsson et al., 2002). Conditional Tcfeb-flox mouse line generation was previously described (Settembre et al., 2012). The liver-specific Tcfeb knockout mouse line was generated by crossing the Tcfeb-flox mouse line with Alb-Cre transgenic mice (Jackson Lab). For all the experiments involving Tcfeb LlKO mice, the control mice were Tcfeb loxP/loxP mice that did not carry the Alb-Cre transgene. All mice used were males (2–3 months old) and maintained on a C57BL/6j strain background. Standard food and water was given ad libitum. For fasting experiments, food pellets were removed from the cages for 24 h. To refeed, the food was added to the cage after 24 h of fasting for 3 h. In the high-fat diet study, age-matched male mice were fed ad libitum a Western-style diet (Harlan Teklad TD 88137) containing 21% (w/w) total lipid (42% calories as anhydrous milk fat). Body weights were recorded every week. The mice were raised under SPF conditions in the animal facility of the Neurological Research Institute (NRI). All experiments were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee (IACUC) and conform to the legal mandates and federal guidelines for the care and maintenance of laboratory animals. The number of mice used is indicated in each figure. The mice were assigned to the experimental groups based on the genotype.

Mice for muscle electroporation experiments were maintained in a temperature- and humidity-controlled animal care facility, with a 12-h light/dark cycle and free access to water and food. All procedures were formerly approved by the Italian Public Health, Animal Health, Nutrition and Food Safety, Italian Ministry of Health (D.M. No. 75/2014-B).

In vivo metabolic analysis

Analysis of oxygen consumption (VO2), carbon dioxide consumption (VCO2) and respiratory exchange ratio (RER) was performed using the Oxymax Columbus Instruments Comprehensive Lab Animal Monitoring System; mice were acclimatized to the system for 24 h before data collection. VO2 and VCO2 data were normalized to lean mass, while energy expenditure data were normalized to body weight. For glucose tolerance tests, mice were injected with glucose (1.5 mg/g body weight) after 6 h of fasting. For insulin tolerance tests, 4-h-fasted mice were injected (intraperitoneal) with insulin (0.75 milliunit/g body weight for Chow diet and 1.0 milliunit/g body weight for HFD, Humulin R; Eli Lilly). For pyruvate tolerance test, mice were injected with sodium pyruvate (2.0 g/kg body weight) after 16-h fasting. Experiments were performed between 10:00 and 12:00. Blood was drawn 15, 30, 60 and 120 min after treatment for determination of glucose and insulin levels. Blood glucose concentrations were measured through tail bleed before and at the times indicated after injection. Muscle glucose uptake has been performed as previously described (Zisman et al., 2000) with a slight modification. For lipolysis studies, we injected 4-h-fasted mice with IP β3-specific agonist CL316243, or a non-specific β agonist isoproterenol (0.1 mg/kg) and collected blood at 0 min (before injection) and 15 min (after stimulation); we determined plasma glycerol and non-esterified fatty acids (NEFA) levels as a measure of lipolysis in vivo (Kyle et al., 2016).

Tissue lipid quantification

Liver triglycerides (TG) were extracted as follows: Briefly, pulverized liver 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 initial homogenate by DC Protein assay (Bio-Rad).

Blood chemistry analysis

Blood was collected from the retro-orbital plexus under isoflurane (Vedco Saint Joseph, MO) anaesthesia. Serum was frozen at −20°C or used immediately after collection. Specific kits were used for the determination of serum lactate (Nova Biomedical), glucagon (Sigma-Aldrich), leptin, adiponectin and insulin (Millipore). Plasma glucose was monitored by a glucometer.

Histology

Livers, epididymal fat (eWAT) and brown adipose tissue (BAT) were dissected, fixed with buffered 10% formalin overnight at 4°C
and stored in 70% ethanol, embedded into paraffin blocks and cut into 6- to 10-μm sections. Tibialis anterior (TA) and gastrocnemius muscles were snap-frozen and cut into 10-μm sections. H&E staining was performed following the IHC World protocol. For Oil Red O staining, OCT-embedded tissues were cut into 25-μm sections, fixed in formalin and stained following the IHC World protocol. For periodic acid-Schiff (PAS) staining, the sections were rehydrated in PBS pH 7.4 and stained using the PAS Kit (Sigma-Aldrich, Saint Louis, MO) following the manufacturer’s instructions. Immunofluorescence staining was performed on 10-μm cryosections as previously described (Sandri et al., 2004; Mammucari et al., 2007) and then monitored with a fluorescence microscope Leica DM5000B equipped with a Leica DFC300-FX digital CCD camera. For nuclear localization studies, cryosections were stained with mouse-anti-dystrophin-1 IgG2a antibodies (Novocastra) and Hoechst to identify the subsarcolemmal position of myonuclei.

Liver glycogen content

For measurements of total glycogen in livers, we used the Glycogen Assay Kit (Cell Biolabs, Inc, San Diego, CA) following the manufacturer's protocol.

Electron microscopy

Liver and muscle specimens were fixed in a mixture of 2% paraformaldehyde and 1% glutaraldehyde prepared in 0.2 M HEPES. Samples were then post-fixed in a mixture of osmium tetroxide and potassium ferrocyanide, dehydrated in ethanol and propylene oxide and embedded in epoxy resin as described previously (Polishchuk et al., 2014). 65-nm-thin sections were cut using a Leica EM UC7 ultramicrotome. EM images were acquired using a FEI Tecnai-12 electron microscope (FEI, Eindhoven, the Netherlands) equipped with a VELETTA CCD digital camera (Soft Imaging Systems GmbH, Munster, Germany). Number of mitochondria was counted using the same magnification within 100-μm square field of view. For each experiment, between 244 and 365 individual mitochondrial diameters were measured from two mice per group.

Acute and chronic exercise

For acute exercise studies, 8-week-old (WT and KO) mice were randomized into three groups: (i) control sedentary (con), (ii) exercise (Ex) and (iii) exercise with 2 h of recovery (Ex+R). Ex and Ex+R groups were run on a treadmill at 10° uphill, to failure. Mice were subjected to a single bout of running starting at the speed of 5 m/min for 5 min, 10 m/min for 10 and 45 min at 15 m/min, and treadmill speed was then increased at a rate of 1 m/min every 2 min until failure. Failure was defined as the point at which the animals could not continue running after three consecutive shocks. Total running time and distance were recorded. Blood lactate and glucose were collected immediately upon exercise cessation and following a 2-h recovery period (Ex+R only). Animals were euthanized immediately after exercise (Ex) or 120 min post-exercise (Ex+R). Muscle was then excised and frozen in liquid nitrogen for later use. For long-term exercise training, WT and KO mice (8 weeks old) were first acclimatized to the treadmill and tested for exercise performance (as described in acute exercise) and glucose tolerance. Mice were then randomly divided into four cohorts, including: (i) mice fed a regular chow diet without daily exercise, (ii) mice fed a HFD containing 42% fat without daily exercise, (iii) mice fed a HFD with daily exercise and (iv) mice fed a HFD without daily exercise. Prior to initiation of exercise, mice were fed a HFD for 4 weeks. At the end of the fourth week, all mice were once again tested for performance. Exercise groups were then trained on the treadmill with 10° uphill incline for 50 min/day, 5 days/week at 10 m/min for 8 weeks. Mice were given a HFD during the 8-week training period. Mouse exercise performance and glucose tolerance were tested one last time at the end of the training protocol.

Animal in vivo transfection experiments

In vivo transfection experiments were performed by intramuscular injection of expression plasmids in tibialis anterior muscle followed by electroporation as previously described (Sandri et al., 2004). Eight days after transfection, mice were exercised on a treadmill. Mice performed exercise on a treadmill (LE 8710 Panlab Technology 2B, Biological Instruments), with a 10° incline, to exhaustion and then were euthanized. Muscle tissue was collected and immediately frozen in liquid nitrogen.

RNA extraction and quantitative PCR

Total RNA was extracted from tissues in TRIzol reagent (Life Technologies, Carlsbad, CA) using RNeasy kit (Qiagen, Hilden, Germany). RNA was reverse-transcribed using a first-strand complementary deoxyribonucleic acid kit with random primers according to the manufacturer’s protocol (Applied Biosystems). The RT–PCRs were performed using CFX96 Real-Time System (Bio-Rad, Hercules, CA). The PCR was performed using the iTaq SYBR Green Supermix (Bio-Rad, Hercules, CA) using the following conditions: pre-heating, 2 minutes at 95°C; cycling, 40 cycles of 15 s at 95°C, 15 s at 60°C and 25 s at 72°C. Results were expressed in terms of relative fold increase in expression levels as determined by ΔΔCt. Primers used for qPCR are listed in Appendix Table S4. Gapdh, Ribosomal protein S16 and β-actin genes were used as endogenous controls (reference markers).

Western blotting

Liver samples were homogenized in RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA pH 8.0, 0.1% SDS) containing Complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Samples were incubated for 20 min at 4°C and centrifuged at 16,000 g for 10 min. The pellet was discarded and cell lysates were used for Western blot analysis. Ten to twenty micrograms of liver protein was electrophoresed on a 4-20% SDS–PAGE polyacrylamide gel. After transfer to nitrocellulose or PVDF membrane, the blots were blocked in TBS–Tween-20 containing 5% non-fat milk for 1 h at RT, and then, primary antibody was applied overnight at 4°C. Anti-rabbit IgG or anti-mouse IgG conjugated with horseradish peroxidase (GE Healthcare, Little Chalfont, UK) and ECL (Pierce, Thermo Fisher Scientific, Wilmington, DE) was used for detection. Antibodies used for immunoblots are listed in Appendix Table S5.
HDAd virus production and injection

HDAd-TFE3 was generated similarly to HDAd-TFEB, as previously described (Pastore et al, 2013; Settembre et al, 2013a). HDAds were produced in 116 cells with the helper virus AdNG163 as described in detail elsewhere (Ng et al, 2002; Palmer & Ng, 2003). Hepatic transduction was achieved by intravenous administration (retro-orbital) of ~200 μl at the dose of 5 × 10^{12} viral particles per kg. Age- and sex-matched mice infected with a transgene deficient HDAd vector served as controls.

Cellular fractionation

Enriched nuclear and cytosolic cellular subfractions were isolated by differential centrifugation, as previously described (Vainshtein et al, 2011). Briefly, the liver was minced on ice and homogenized using a Teflon pestle and mortar and suspended in mitochondrial isolation buffer (MIB; 250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl_2, 1 mM EDTA, 1 mM EGTA) supplemented with protease and phosphatase inhibitor cocktails (Complete and PhosSTOP Roche, Roche Diagnostics, Basel, Switzerland). The homogenates were then centrifuged at 1,000 g for 10 min at 4°C to pellet the nuclei while mitochondrial and cytosolic fractions were contained within the supernatant. The supernatant fraction was re-centrifuged twice at 16,000 g for 20 min at 4°C to pellet the mitochondria and supernate containing cytosolic subfraction was collected. Pellets containing nuclei were re-suspended in nuclear lysis buffer (1.5 mM MgCl_2, 0.2 mM EDTA, 20 mM HEPES, 0.5 M NaCl, 20% glycerol, 1% Triton X-100), incubated on ice for 30 min, and then sonicated 3 × 10 s followed by a final centrifugation step of 15 min at 16,000 g. The supernatant was collected to obtain the enriched nuclear fraction. Fraction purity was determined by Western blot analysis.

Identification of TFE3 mitochondrial target genes

The TFE3 target genes list was obtained by Betschinger et al (2013). The list of well-known mitochondrial genes was downloaded from the Molecular Signatures Database (MsigDB; Liberzon et al, 2011; Liberzon, 2014). 136 mitochondrial genes were found to be direct targets of TFE3. The significance of the enrichment of the mitochondrial genes into the TFE3 target genes list was calculated by using the hypergeometric test (P-value = 2.38E-23). The analysis of the biological processes was performed by using DAVID online tool (DAVID Bioinformatics Resources 6.7; Dennis et al, 2003; Huang da et al, 2009).

Cell culture, plasmids and transfection reagents

Primary mouse hepatocytes were isolated using a two-step perfusion technique as previously described (Seglen, 1976). Briefly, WT or KO mouse liver was perfused with collagenase (CS5138, Sigma-Aldrich, Saint Louis, MO) and parenchymal cells were squeezed from the liver. Hepatocyte suspension were further purified by 40% Percoll gradient (P4937, Sigma-Aldrich, Saint Louis, MO), washed with hepatocyte wash medium (17004-024; Thermo Fisher Scientific) and plated in an appropriate cell density. Cells were harvested after 24 h for analysis. For immunofluorescence, cells were fixed in 4% PFA for 20 min, washed twice in PBS, permeabilized in PBS–Triton 0.01% and incubated with the primary antibody overnight at 4°C. The next day, the cells were washed in PBS and incubated with the secondary antibody for 1 h. Coverslips were mounted using the ProLong® Gold Antifade Reagent with DAPI (Invitrogen).

HeLa CRISPR/Cas9 cells were kindly provided by R. Youle (National Institute of Health, NIH) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37°C. Wild-type and Tfe3 KO murine embryonic fibroblasts (MEFs) were isolated from embryos at E13.5 following previously established protocols and cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37°C.

For ROS assessment, MEFs were transfected with Matrix-roGFP plasmid, a gift from Paul Schumacker (Waypa et al, 2010; Addgene plasmid # 49437). Cells were transfected using Lipofectamine LTX reagent (Invitrogen) according to the manufacturer’s instructions.

Mitochondrial membrane potential analyses

Mitochondrial membrane potential was measured in Hela, MEFs and isolated hepatocytes. Mitochondrial membrane potential was measured by epifluorescence microscopy based on the accumulation of TMRM fluorescence as previously described (Romanello et al, 2010).

Seahorse XF-24 metabolic flux analysis

Oxygen consumption was measured at 37°C using an XF-24 extra-cellular analyser (Seahorse Bioscience Inc., North Billerica, MA, USA). Primary hepatocytes (2 × 10^5) were seeded in 24-well plates. After 24 h, the medium was replaced with unbuffered DMEM containing 5 mM glucose and 1 mM pyruvate and the cells were incubated at 37°C without CO_2 for 1 h. Substrates were added at a final concentration of oligomycin 1 μM, FCCP 1 μM and antimycin A 4 μM. The experiment was performed in hepatocytes isolated from 4 WT and 4 Tfe3 KO mice. Each data point represents an average of 10 different wells. Mitochondria were isolated from WT and Tfe3 KO mice as previously described (Frezza et al, 2007). 15 μg of mitochondria were plated on a 24-well plate in MAS buffer (70 mM sucrose, 220 mM mannitol, 5 mM KH_2PO_4, 5 mM MgCl_2, 2 mM HEPES, 1 mM EGTA, 0.2% fatty acid-free BSA) supplemented with 2 μM rotenone and 10 mM succinate and centrifuged for 20 min at 2,000 g at 4°C. Mitochondria were incubated at 37°C without CO_2 for 10 min. Substrates were added at a final concentration of ADP 1 mM, oligomycin 2 μM, FCCP 4 μM and antimycin A 4 μM.

Chromatin immunoprecipitation (ChiP)

ChiP was performed using livers of 2-month-old WT control and WT injected with HDAd-TFE3 virus 1 month after injection and Tfe3 KO mice as previously described (Settembre et al, 2013a). Primers for a different E-Box not target for TFE3 have been used as negative control. Primers used:

5′-GCCCTTTAATCCAGCAATCA-3′ Fis1 E-Boxes Forward
5′-ATACCCCTCTCCATGCTCAC-3′ Fis1 E-Boxes Reverse
5′-TGCTTGTTTTATTCTG-3′ Ctrl E-Box Forward
5′-AAGTTAGAGGCTAGACTGAGG-3′ Ctrl E-Box Reverse
The paper explained

Problem
The mechanisms by which complex organisms adapt their energy metabolism to environmental cues are not fully understood. Elucidation of such adaptation mechanisms, and identification of the metabolic pathways involved, is of pivotal importance for the understanding of human physiology and disease pathogenesis.

Results
We demonstrated that the transcription factors TFE3 and TFEB regulate lipid and glucose metabolism and mitochondrial biogenesis, and play a cooperative role in the control of the adaptive response of whole-body metabolism to environmental cues such as diet and physical exercise.

Impact
Our study reveals the importance of TFE3 and TFEB in the metabolic response to environmental cues and in patholgical conditions associated with defective glucose and lipid metabolism. Indeed, these transcription factors may represent novel therapeutic targets for diet-induced obesity and diabetes.

Statistical analyses

Obtained data were processed in Excel (Microsoft Corporation) and Prism (GraphPad Software) to generate bar charts and perform statistical analyses. Data are expressed as mean values ± standard error (SE). Statistical significance was computed using Student’s two-tailed t-test or ANOVA as reported in the figure legends.

Expanded View for this article is available online.

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Author contributions
NP and AV performed most of the experiments. TKJ performed the ChIP experiment. AA performed the muscle electroporation experiments. TH and NJH provided technical support to NP and AV. EVP performed the EM analysis. MS contributed to the interpretation of the results. AB and NP designed the overall study, supervised the work and wrote the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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