Review

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The complex relationship between TFEB transcription factor phosphorylation and subcellular localization

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Abstract

The MiT-TFE family of basic helix-loop-helix leucine-zipper transcription factors includes four members: TFEB, TFE3, TFEC, and MITF. Originally described as oncogenes, these factors play a major role as regulators of lysosome biogenesis, cellular energy homeostasis, and autophagy. An important mechanism by which these transcription factors are regulated involves their shuttling between the surface of lysosomes, the cytoplasm, and the nucleus. Such dynamic changes in subcellular localization occur in response to nutrient fluctuations and various forms of cell stress and are mediated by changes in the phosphorylation of multiple conserved amino acids. Major kinases responsible for MiT-TFE protein phosphorylation include mTOR, ERK, GSK3, and AKT. In addition, calcineurin de-phosphorylates MiT-TFE proteins in response to lysosomal calcium release. Thus, through changes in the phosphorylation state of MiT-TFE proteins, lysosome function is coordinated with the cellular metabolic state and cellular demands. This review summarizes the evidence supporting MiT-TFE regulation by phosphorylation at multiple key sites. Elucidation of such regulatory mechanisms is of fundamental importance to understand how these transcription factors contribute to both health and disease.

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Introduction

Transcriptional regulation plays a crucial role in the adaptation of cell homeostasis to environmental cues. Nucleo-cytoplasmic shuttling of transcription factors is used by the cell to control gene expression programs in response to the environment. Transcription factors exert their function primarily in the nucleus; however, under some conditions, they may be localized in the cytoplasm. The import of transcription factors from the cytoplasm to the nucleus is facilitated by several mechanisms, importin-mediated and importin-independent (Xu & Massague, 2004). For example, the NF-κB and NFAT transcription factors are imported to the nucleus through a nuclear localization signal (NLS), a cluster of basic amino acids that is recognized by importin-β, which in turn binds importin-α to promote nuclear import. In contrast, nuclear translocation of SMAD transcription factors is importin-independent and requires their direct binding to the nuclear pore (Beg *et al*, 1992; Henkel *et al*, 1992; Zhu *et al*, 1998; Hill, 2009). This process is dependent on the activity of the small GTPase Ran, which mediates nuclear import and export of transcription factors (Gorlich & Kutay, 1999; Xu & Massague, 2004).

Extracellular signals may affect nucleo-cytoplasmic shuttling of transcription factors in several ways. A common mechanism used by the cell to link signaling pathways to the control of gene expression is the phosphorylation of transcription factors (Nardozzi *et al*, 2010). One mode of regulation by phosphorylation involves controlling protein subcellular localization. Phosphorylation may promote nuclear import by enhancing the binding affinity for importins or by unmasking an NLS, but may also inhibit nuclear import by acting on a component of the nuclear transport machinery or by disrupting the NLS (Nardozzi *et al*, 2010). In the case of transcription factors, the control of their subcellular localization is an important way to modulate gene expression programs with profound effects on the metabolic adaptation to environmental cues.

In this review article, we focus on how phosphorylation-mediated signaling pathways regulate the subcellular localization and function of transcription factor EB (TFEB) and the other members of the MiT-TFE family. Such regulation is of importance

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for coordinating expression of lysosomal-autophagic pathway and cell metabolism genes and thereby allowing cells to adapt to changing environmental cues.

Transcription factor EB and the MiT-TFE family

The MiT-TFE family

The MiT-TFE family of transcription factors includes four members: MITF, TFEB, TFE3, and TFEC (Steingrimsson *et al*, 2004). These are helix-loop-helix (HLH) leucine-zipper transcription factors that share high sequence similarities and activate expression of their target genes by binding DNA either as homo- or hetero-dimers. Like other members of the larger family of HLH leucine-zipper transcription factors, MiT-TFE proteins bind a palindromic DNA sequence (CACGTG) located in the proximal promoter of target genes (Fisher *et al*, 1991; Hemesath *et al*, 1994). This sequence, referred to as an E-box, conforms to the CANNTG motif that is recognized by other members HLH/leucine-zipper family transcription factors (Hemesath *et al*, 1994). However, specificity for DNA binding in the HLH-LZ family is influenced by sequences immediately flanking the E-box such that the MiT-TFE proteins prefer the GTCACGTGAC consensus sequence that is known as a CLEAR motif (Sardiello *et al*, 2009; Palmieri *et al*, 2011).

Structural and biochemical data suggest that MiT-TFE proteins may heterodimerize with one another but not with other members of the HLH/leucine-zipper family (Pogenberg *et al*, 2012). MiT-TFE genes are present in all metazoan organisms. However, commonly studied invertebrates such as *Drosophila* and *Caenorhabditis elegans* only have a single member of the family, named *Mitf* and HLH-30, respectively (Rehli *et al*, 1999; Hallsson *et al*, 2004), whose function and regulation appear to be similar to TFEB (Lapierre *et al*, 2013; O'Rourke & Ruvkun, 2013; Settembre *et al*, 2013b; Zhang *et al*, 2015; Bouche *et al*, 2016; Tognon *et al*, 2016). Proteins of the MiT-TFE family have a large degree of overlap in their function and regulatory mechanisms. In general, they are ubiquitously expressed but their expression levels in different tissues vary considerably. One exception is an MITF splice variant (MITF-M) that exhibits constitutive nuclear localization and is expressed almost exclusively in melanocytes (Yasumoto *et al*, 1998).

TFEB and TFE3 as master regulators of lysosomal function and autophagy

While the function of MITF as regulator of melanoblast survival and differentiation, melanosome biogenesis, and eye development has been known for some time due to the striking coat color and eye development defects detected in mice and rats harboring spontaneous MITF mutations (Hodgkinson *et al*, 1993; Steingrimsson *et al*, 1994, 2004; Opdecamp *et al*, 1997), the function of the other members of the MiT-TFE family has been more elusive. Notably, while TFEB knock-out mice are embryonic lethal (Steingrimsson *et al*, 1998), there was an apparent lack of overt phenotypes in both TFE3 and TFEC knock-out mice (Steingrimsson *et al*, 2002).

Important insights into the function of TFEB, and subsequently of its closely related paralogue TFE3, came from a systems biology study aimed at testing the hypothesis that lysosomal function was globally regulated at the transcriptional level. This study led to the identification of a transcriptional network of genes involved in lysosomal biogenesis, named coordinated lysosomal expression and regulation (CLEAR) network, and of TFEB as its master regulator (Sardiello *et al*, 2009). Subsequent studies demonstrated that TFEB is also able to regulate multiple aspects of lysosome function such as autophagy (Settembre *et al*, 2011) and lysosomal exocytosis (Medina *et al*, 2011). Interestingly, TFE3 was also found to control lysosomal biogenesis and autophagy by regulating a gene network that largely overlaps with the one regulated by TFEB (Martina *et al*, 2014).

The role of TFEB in the control of lysosomal biogenesis, autophagy, and lysosomal exocytosis may be exploited to promote cellular clearance in a number of disease conditions (Medina *et al*, 2011). This approach was tested in several cellular and mouse models of human diseases resulting from the accumulation of undegraded substances, such as lysosomal storage diseases (LSD; Medina *et al*, 2011; Song *et al*, 2013; Spampanato *et al*, 2013; Rega *et al*, 2016), Parkinson's disease (Dehay *et al*, 2010; Decressac *et al*, 2013; Kilpatrick *et al*, 2015), Alzheimer's disease (Polito *et al*, 2014; Xiao *et al*, 2014; Chauhan *et al*, 2015), and diet-induced obesity (Settembre *et al*, 2013a), among others. Like TFEB, TFE3 was shown to promote cellular clearance in a mouse model of Pompe disease (Martina *et al*, 2014).

Physiological roles of TFEB and TFE3

Loss-of-function approaches based on knock-out (KO) mice have been used to explore the physiological roles of MiT-TFE genes. Single and double KO mice were used to investigate redundancy and cooperation between these transcription factors. Interestingly, double KO mice for MITF and TFE3 show osteoclast defects that were not observed in either of the respective single KO strains (Steingrimsson *et al*, 2002). Due to the embryonic lethality of global TFEB KO mice, which arises due to a defect in placental vascularization (Steingrimsson *et al*, 1998), conditional KO mice were generated in which TFEB was deleted in specific tissues. Table 1 lists the TFEB full KO line and tissue-specific conditional KO and conditional overexpressor lines that were generated to explore the physiological role of TFEB in specific organs and tissues. TFEB liver-specific conditional KO mice displayed severe abnormalities of lipid metabolism that are significantly enhanced by a high-fat diet, resulting in severe obesity and diabetes (Settembre *et al*, 2013a; Pastore *et al*, 2017). This effect of TFEB on lipid metabolism is mediated by genes involved in lipid degradation pathways, such as fatty acid oxidation and lipophagy (Settembre *et al*, 2013b).

Loss of TFEB in the muscle has a major impact on cellular energy metabolism. TFEB muscle-specific conditional KO mice showed impaired glucose homeostasis and mitochondrial biogenesis with decreased fatty acid oxidation and oxidative phosphorylation. In addition, these mice have reduced metabolic flexibility during physical exercise, which makes them unable to exploit its beneficial effects on metabolism. Transcriptomic analysis of muscle-specific TFEB KO mice showed that in muscle, TFEB regulates the expression of genes encoding glucose transporters and glycolytic enzymes as well as genes involved in mitochondrial biogenesis (Mansueto *et al*, 2017).

Transcription factor EB was also shown to play a broad role in the regulation of the innate immune response. A key signaling pathway is the cGAS-STING pathway, which senses double-stranded DNA (dsDNA) in the cytosol and leads to the activation of an

Table 1. TFEB full KO line and tissue-specific conditional KO and conditional overexpression lines that were generated to explore the physiological role of TFEB in specific organs and tissues.

Mouse line	Tissue	Phenotype	References
Loss of function			
Full KO	Ubiquitous	Embryonic lethality (E9.5)	Steingrimsson *et al* (1998)
Conditional KO	Bone (*CtsK-Cre*)	Defective bone resorption	Ferron *et al* (2013)
Conditional KO	Liver (*Alb-Cre*)	Impaired liver metabolism, metabolic imbalance, exacerbated obesity	Settembre *et al* (2013a) and Pastore *et al* (2017)
Conditional KO	Macrophages (*Lys2-Cre*)	Impaired inflammatory and immune response	Pastore *et al* (2016)
Conditional KO	Muscle (*Mlc1f-Cre*)	Impaired mitochondrial function and glucose homeostasis	Mansueto *et al* (2017)
Conditional KO	Intestinal epithelium (*Villin-Cre*)	Exacerbated colitis	Murano *et al* (2017)
Conditional KO	Endothelial cells (*VE-Cad-Cre*)	Decreased angiogenesis and attenuated blood flow recovery after ischemic injury	Fan *et al* (2018)
Gain of function			
Transgenic	Brain (*Thy1* promoter)	Clearance of toxic aggregates in an AD model	Wang *et al* (2016)
Conditional transgenic	Muscle (*HSA-Cre-Er*)	Increased glucose and lipid metabolism	Mansueto *et al* (2017)
Conditional transgenic	Kidney (*Cdh16-Cre*; *Cdh16-Cre-Ert2*)	Renal cystic pathology and papillary carcinoma	Calcagni *et al* (2016)
Conditional transgenic	Macrophages (LyzM-Cre)	Enhanced degradative capacity, reduced atherosclerosis in ApoE null mice	Sergin *et al* (2016)
Transgenic	Endothelial cells (*Tie2* promoter)	Increased angiogenesis and improved blood flow recovery after ischemic injury	Fan *et al* (2018)

inflammatory response. Indeed, inactivation of TREX1, an exonuclease, leads to cytosolic DNA accumulation, TFEB nuclear localization and increased lysosome biogenesis (Hasan *et al,* 2013). In addition, TFEB plays a role in activated macrophages. Conditional KO mice lacking TFEB in macrophages showed decreased expression of genes encoding pro-inflammatory cytokines and chemokines. Furthermore, macrophages lacking TFEB displayed impaired autophagy and lysosomal biogenesis (Pastore *et al*, 2016). Such lysosomal defects arising from TFEB depletion in macrophages have furthermore been shown to impair their ability to upregulate antibacterial activities in response to bacterial exposure (Gray *et al*, 2016). Further evidence for the role of TFEB in inflammation and immunity came from the study of conditional KO mice in which TFEB was specifically deleted in the intestinal epithelium, which showed increased susceptibility to epithelial cell injury and subsequent colitis (Murano *et al*, 2017). Recently, by using endothelial cell (EC)-specific TFEB transgenic and KO mice, TFEB was also shown to positively regulate angiogenesis via activation of AMPK and autophagy (Fan *et al,* 2018).

Remarkably, TFE3 full KO mice appeared superficially healthy, but in-depth analysis revealed cellular and metabolic phenotypes that are very similar to TFEB liver-, muscle-, and macrophage-specific conditional KO mice, and such effects are significantly enhanced by the loss of both TFEB/TFE3 in these tissues (Pastore *et al*, 2016, 2017). These data suggest that these two transcription factors regulate very similar sets of genes in multiple tissues and play a cooperative, rather than redundant role. Alternatively, the lack of redundancy may reflect a high degree of sensitivity to their dosage. Consistent with the idea of dosage, TFEB overexpression via viral-mediated gene transfer was able to rescue the phenotype of TFE3 KO mice and vice versa (Pastore *et al*, 2017).

Recently, TFEB and TFE3 were also shown to regulate the induction of protein synthesis via mechanistic target of rapamycin complex 1 (mTORC1) upon amino acid feeding after starvation or physical exercise. This effect is mediated by the transcriptional regulation of RagD GTPase and is important for an efficient mTORC1 recruitment to the lysosomal surface (Di Malta *et al*, 2017).

Role of MiT-TFE transcription factors in cancer

Transcription factor EB and other members of the MiT-TFE family of transcription factors have been known for quite some time for their oncogenic features (Kuiper *et al*, 2003; Haq & Fisher, 2011; Kauffman *et al*, 2014). Chromosomal translocations involving the TFEB and TFE3 genes cause a particular type of renal cancer, referred to as translocation carcinoma (Linehan *et al*, 2010; Malouf *et al*, 2014). These fusions consistently preserve the TFEB/TFE3 open reading frame and always include the DNA-binding domains (Kuiper *et al*, 2003). While a variety of genes can be fused to TFEB or TFE3 a major consequence of the translocation with respect to oncogenic activity is a massive increase in the expression levels of a TFEB or TFE3 protein. This conclusion is supported by translocations that increase TFEB expression without changing the coding sequence (Kuiper

et al, 2003). Interestingly, MITF may also be translocated in tRCC (Durinck *et al*, 2015). tRCCs account for approximately 1–5% of all RCCs, but are particularly prevalent in children and adolescents. TFE3, which is the most common member translocated in tRCC, is also translocated in a particular type of sarcoma (alveolar soft part sarcoma) and in perivascular epithelioid tumors (PEComas).

The ASPSCR1-TFE3 translocation, which is characteristic of alveolar soft part sarcoma and also frequently observed in tRCC, was modeled in mice (Goodwin *et al*, 2014). A human cDNA fusion was targeted to the *Rosa26* locus, and its expression was activated using both a tamoxifen-inducible Cre-recombinase (CreER) also from the *Rosa26* locus and a Prx1-CreER^{T2}. Cre expression in both models removed a stop signal leading to expression of the ASPSCR1-TFE3 fusion protein. Prx1 is expressed in osteochondral progenitors, neural stem cells, and in some intramuscular pericytes. In the CreER-driven mice, stochastic, low-level expression (in the absence of tamoxifen) resulted in tumors in the brain, choroid plexus, and orbit between 3 and 6 months. Prx1-CreER^{T2} mice developed tumors in the brain and intracranial periosteum following tamoxifen administration. No skeletal muscle (or kidney) tumors were reported in either strain. Overall, while the anatomic sites markedly differed between human and murine tumors, the murine tumors expressed the fusion cDNA and resembled alveolar soft part sarcomas both histologically and by gene expression. The authors speculate that the sites of tumor development in the mice are the result of a microenvironment rich in lactate, which is used by the tumors as a source of energy.

In addition to translocation, TFEB may also induce renal cancers through amplification (Durinck *et al*, 2015). Interestingly, the *TFEB* gene resides in the proximity of *VEGFA* and both genes may be amplified together (Gupta *et al*, 2017). Recently, a kidney-specific TFEB-overexpressing mouse line was generated using a Cadherin16 Cre (or CreER^{T2}), which recapitulates some features of human TFEB/TFE3-associated RCC (Calcagni *et al*, 2016). These mice developed renal cysts and papillary carcinomas, followed by liver metastases. This mouse model shows TFEB-/TFE3-dependent induction of both the Wnt-beta-catenin and mTORC1 pathways (Calcagni *et al*, 2016; Di Malta *et al*, 2017).

MITF, TFEB, and TFE3 overexpression was also observed in pancreatic adenoductal carcinoma, where they appear to support tumor growth via the induction of autophagy (Perera *et al*, 2015). MITF is an established oncogene and can be found amplified in a subset of melanoma patients (Tsao *et al*, 2012). Interestingly, tumors in which MiT-TFE genes are amplified or overexpressed show an induction of RagD, a direct transcriptional target of TFEB, that resulted in mTORC1 hyperactivation (Di Malta *et al*, 2017). The RagD GTPase is important for an efficient mTORC1 recruitment to the lysosomal surface. Silencing of MiT-TFE genes in primary cell cultures obtained from tumors resulted in a significant reduction in the hyperproliferative phenotype. Furthermore, xenotransplantation experiments performed using a melanoma cell line showed that silencing of RagD significantly reduced tumor growth (Di Malta *et al*, 2017). In addition to promoting various cancers, TFEB was also recently identified as being highly expressed in non-Hodgkins lymphoma where it may render these cancer cells sensitive to apilimod-mediated inhibition of the PIKfyve lipid kinase (Gayle *et al*, 2017).

Transcription factor EB subcellular localization

Conditions that promote TFEB nuclear translocation

Initial evidence for the shuttling of TFEB between the cytoplasm and the nucleus was obtained in cells treated with sucrose, which is endocytosed and accumulated in the lysosomes due to their lack of invertase enzymes, and thus provides a cellular model of lysosome storage (Sardiello *et al*, 2009). While TFEB was primarily localized in the cytoplasm in untreated cells, sucrose treatment was followed by relocation of TFEB from the cytoplasm to the nucleus. Other pharmacological treatments that result in lysosome stress such as chloroquine (a weak base that neutralizes lysosome pH), bafilomycin or trehalose, also cause TFEB nuclear translocation (Roczniak-Ferguson *et al*, 2012; Settembre *et al*, 2012; Palmieri *et al*, 2017). Both TFE3 and MITF are also subject to nucleo-cytoplasmic shuttling and behave similarly to TFEB (Bronisz *et al*, 2006; Roczniak-Ferguson *et al*, 2012; Martina & Puertollano, 2013; Martina *et al*, 2014).

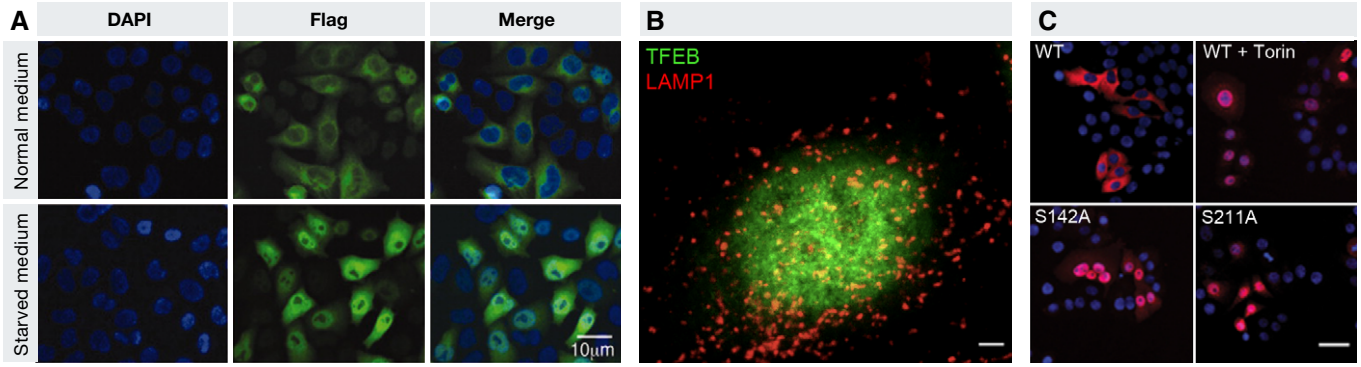

Figure 1. TFEB subcellular localization is both nutrient- and phosphorylation-dependent.
(A) HeLa cells cultured in either normal or starved medium were stained for Flag-TFEB (green; Settembre *et al*, 2011). (B) 3D reconstruction of Airyscan super-resolution imaging of HeLa cells stained for TFEB (green) and LAMP1 (red; Image courtesy of JIenia Monfregola and A.B.). (C) TFEB phosphorylation of specific serine residues controls its subcellular localization. Flag immunostaining (red) of HeLa cells transfected with WT TFEB and treated with Torin 1 and of HeLa cells transfected with serine-to-alanine mutated versions of 3xFlag-TFEB and grown in normal medium (Settembre *et al*, 2012).

A variety of stimuli have been associated with TFEB nuclear translocation, the best studied of which is nutrient deprivation. When cells are grown in starvation medium, TFEB relocates to the nucleus as early as 1 h after exposure to the nutrient-deprived (amino acid and serum) medium (Settembre *et al*, 2011, 2012; Martina *et al*, 2012; Roczniak-Ferguson *et al*, 2012). Figure 1A shows a cytoplasmic localization of TFEB in HeLa cells grown in normal medium and a nuclear localization in cells grown in nutrient-deprived medium. A nuclear localization of TFEB is also evident in Fig 1B, which shows a super-resolution image of a HeLa cell stably transfected with a TFEB-GFP plasmid and grown with a nutrient-deprived medium. This observation appears to be consistent in all cell types studied. *In vivo* studies also demonstrated that starvation (i.e., elimination of the food) for approximately 16 h in mice results in a nuclear localization of TFEB in all tissues studied (Settembre *et al*, 2011; Chen *et al*, 2017). In cell culture, re-feeding after starvation results in nucleus-to-cytoplasm re-localization of TFEB within minutes (Settembre *et al*, 2011, 2012; Martina *et al*, 2012; Roczniak-Ferguson *et al*, 2012). A similar acute behavior was observed for TFE3 (Martina *et al*, 2014).

This acute sensitivity of MiT-TFE proteins to nutrient availability reflects their dual dependence on the lysosome-localized and amino acid-regulated Rag GTPases: first, as TFEB-Rag interactions underlie the recruitment the transcription factor to the surface of the lysosomes (Martina & Puertollano, 2013), and second, as Rags are also responsible for communicating amino acid availability to mTORC1 (Sancak *et al*, 2010, 2008). Impaired control of the nuclear versus cytoplasmic localization of TFEB by Rag GTPases may also contribute to human disease. For example, loss-of-function mutations in folliculin (FLCN), a GTPase-activating protein (GAP) for RagC/D, result in constitutively nuclear localization of TFEB and TFE3 and give rise to Birt-Hogg-Dube' syndrome, a disease characterized by benign, hair follicle tumors (fibrofolliculomas), risk of pneumothorax, and frequent occurrence of bilateral, multifocal renal carcinoma (Hong *et al*, 2010; Petit *et al*, 2013; Tsun *et al*, 2013; Schmidt & Linehan, 2015).

In addition to lysosomal storage and starvation, other conditions were found to promote TFEB nuclear localization. These include infection (Visvikis *et al*, 2014; Campbell *et al*, 2015; Pastore *et al*, 2016), bacterial phagocytosis (Gray *et al*, 2016), inflammation (i.e., LPS treatment; Pastore *et al*, 2016), physical exercise (in muscle; Mansueto *et al*, 2017), mitochondrial damage (Nezich *et al*, 2015), PIKfyve inhibition (Gayle *et al*, 2017), and ER stress (Martina *et al*, 2016). In addition, inactivation of the cytosolic exonuclease, TREX1, also leads to TFEB nuclear localization and lysosome expansion (Hasan *et al*, 2013). It is becoming evident that multiple types of cellular stress ultimately induce TFEB nuclear localization, suggesting that TFEB subcellular localization is controlled by a stress response mechanism (Raben & Puertollano, 2016). It remains to be determined, however, to what extent these various stressors impinge on MiT-TFE proteins via mTOR-dependent versus mTOR-independent mechanisms (see below).

It is important that appropriate, and ideally multiple, methods are used to test the effects of a specific condition on TFEB subcellular localization. The most commonly used methods are immunofluorescence and nucleo-cytoplasmic fractionation. These methods can be used on both endogenous and ectopically overexpressed TFEB (Settembre & Medina, 2015). The low abundance of TFEB, particularly in some cell types, has hampered the analysis of the endogenous protein. Therefore, it is particularly important to use high-quality antibodies. Due to the difficulties to analyze endogenous TFEB, many studies have relied on cells stably transfected with tagged versions of TFEB. However, when TFEB is overexpressed at high levels, a significant portion of the protein can be found in the nucleus even in normally fed cells.

When using immuno-fluorescence, it is particularly important to analyze many cells in order to get robust and statistically significant results. This can be achieved by using high-content analysis with an automated confocal microscope (Settembre & Medina, 2015). This system allows for a completely unbiased analysis of hundreds of cells. It may also be helpful to score not only cells with nuclear or cytoplasmic TFEB, but also ratios of nuclear to cytoplasmic localization in cells with a mixed pattern where TFEB is found in both compartments (Petit *et al*, 2013). It is also worth considering that while changes in the subcellular distribution suggest alterations in TFEB shuttling from one compartment to the other, compartment-selective degradation has not been excluded in most contexts and may give the same results. Another aspect to consider when seeking to understand the regulation of endogenous MiT-TFE family members in diverse cell types is the variability in their relative expression levels such that different family members may predominate in a given cell type.

mTOR-mediated TFEB phosphorylation

The best-studied mechanism that regulates TFEB subcellular localization involves the phosphorylation of specific serine residues in the TFEB protein. Other, phosphorylation-independent, mechanisms have been proposed but never formally demonstrated. Table 2 lists all TFEB and TFE3 phosphorylation sites that have been directly evaluated to date. The mTOR kinase was shown to phosphorylate specific serine residues in TFEB and to play a major role in the regulation of TFEB subcellular localization. The nutrient dependence of mTOR-mediated TFEB phosphorylation indicated that mTORC1 was the complex involved (Settembre *et al*, 2011, 2012; Martina *et al*, 2012; Roczniak-Ferguson *et al*, 2012; Vega-Rubin-de-Celis *et al*, 2017). At least three serines, S122, S142, and S211, in the TFEB protein are phosphorylated by mTORC1 (Settembre *et al*, 2011, 2012; Martina *et al*, 2012; Roczniak-Ferguson *et al*, 2012; Vega-Rubin-de-Celis *et al*, 2017). Mutations of either S142 or S211 into alanines (S142A, S211A) result in a constitutively nuclear TFEB, similar to cells treated with the mTOR inhibitor Torin 1, as shown in Fig 1C.

Phosphorylation of S211 determines TFEB binding with 14-3-3 protein. It has been hypothesized that this binding masks an NLS, thus inhibiting TFEB nuclear translocation (Martina *et al*, 2012; Roczniak-Ferguson *et al*, 2012). A recent study also showed that the phosphorylation of serines S142 and S211 also mediates the targeting of TFEB to the ubiquitin proteasome system via the binding to the E3 ubiquitin ligase STUB1, suggesting that phosphorylation may regulate TFEB function not only by determining its subcellular localization but also by modulating its stability (Sha *et al*, 2017).

In vitro kinase assays showed that S122 is directly phosphorylated by mTORC1 (Vega-Rubin-de-Celis *et al*, 2017). Mutation of S122 to alanine (S122A) does not, by itself, affect TFEB subcellular localization but appears to enhance the effects of the S211A mutation (Vega-Rubin-de-Celis *et al*, 2017). However, the phosphomimetic mutation of S122 to aspartic acid (S122D) blocks the effects of the S211A mutation on TFEB nuclear translocation. Nevertheless, whether the mechanism whereby aspartate

Table 2. TFEB and TFE3 phosphorylation sites that have been directly evaluated to date.

Site	Kinase	Method used to characterize site	Treatments	Effects of site phosphorylation on TFEB	References
TFEB					
S122	mTOR	*In vitro* kinase assay, phospho-antibody, mutation of modification site, Western blotting, immunoprecipitation	Amino acid starvation, glucose starvation, serum starvation, Torin-1	Cytoplasmic retention	Vega-Rubin de Celis *et al* (2017)
S134/S138	GSK3B	*In vitro* kinase assay, mutation of modification site	GSK3 inhibitors, PMA, angiotensinII, LPS	Cytoplasmic retention	Li *et al* (2016)
S142	Erk 1/2, mTOR	Phospho-antibody, *in vitro* kinase assay, mutation of modification site, Western blotting	Amino acid starvation, serum starvation, Torin-1, antimycinA/ oligomycin	Cytoplasmic retention	Settembre *et al* (2011), Settembre *et al* (2012), Nezich *et al* (2015)
S211	mTOR	*In vitro* kinase assay, mass-spectometry, phospho-antibody, mutation of the modification site, Western blotting, immunoprecipitation	Amino acid starvation, serum starvation, lysosomal stress, Torin-1/PP42	Cytoplasmic retention, 14-3-3 binding	Martina *et al* (2012), Roczniak-Ferguson *et al* (2012), Settembre *et al* (2012), Vega-Rubin de Celis *et al* (2017)
S462/S463/S467/S469	PKCB	Mutation of modification site, Western blotting	RANKL	Protein stabilization	Ferron *et al* (2013)
S462/S463/S466/S467/ S469	Hyperactive mTOR ($TSC2^{-/-}$ cells)	Mutation of modification site	Rapamycin, serum starvation	Nuclear translocation	Pena-Llopis *et al* (2011)
S467	AKT	*In vitro* kinase assay, mutation of modification site	Trehalose	Cytoplasmic retention	Palmieri *et al* (2017)
TFE3					
S321	mTOR	*In vitro* kinase assay, phospho-antibody, mutation of modification site, Western blotting, immunoprecipitation	Amino acid starvation, serum starvation, Torin-1, tunicamycin, LPS	Cytoplasmic retention, 14-3-3 binding	Martina *et al* (2014), Martina *et al* (2016), Pastore *et al* (2016), Wada *et al* (2016)

substitution affects TFEB localization is by mimicking phosphorylation remains to be determined.

The mechanisms by which the phosphorylation of S142 and S122 affect TFEB subcellular localization are still unclear. TFE3 subcellular localization is also regulated by mTORC1-mediated phosphorylation and involves serine residues that are conserved between TFEB and TFE3 (Martina *et al*, 2014, 2016; Wada *et al*, 2016). Data obtained from patient-derived pancreatic cancer cell lines revealed that TFEB and TFE3 nuclear translocation is mediated by specific importins; however, it is still unclear whether this is a general mechanism relevant in all cell types (Perera *et al*, 2015).

It is particularly important to test the phosphorylation of TFEB by immunoblotting using standard antibodies directed to TFEB (i.e., looking at the TFEB electrophoretic mobility on SDS–PAGE gels, which is influenced by phosphorylation) and phosphospecific antibodies that recognize the phosphorylation state of specific amino acid residues. Given how widely phosphorylation occurs and the difficulties related to generating highly quality phosphospecific antibodies, their validation using phosphosite mutants (i.e., alanine substitution of the particular site) is advisable. With respect to TFEB, phosphospecific antibodies that detect phosphorylation on S142 and S211 have been reported (Settembre *et al*, 2011; Petit *et al*, 2013). An alternative method to measure phosphorylation on S211 that confers binding capabilities to 14-3-3 proteins when in the phosphorylated state is the use of an anti-14-3-3-binding motif antibody on TFEB immunoprecipitates (Martina *et al*, 2012; Roczniak-Ferguson *et al*, 2012). Phosphoproteomic studies have revealed that TFEB is phosphorylated at multiple sites (more than 20; Dephoure *et al*, 2008; Chen *et al*, 2009; Mayya *et al*, 2009; Huttlin *et al*, 2010; Olsen *et al*, 2010), many of which are phosphorylated by mTOR (Yu *et al*, 2011). Figure 2 shows all the serine residues in the TFEB protein that were shown to be subject to phosphorylation by functional studies. It is worth noting that key residues and regulatory

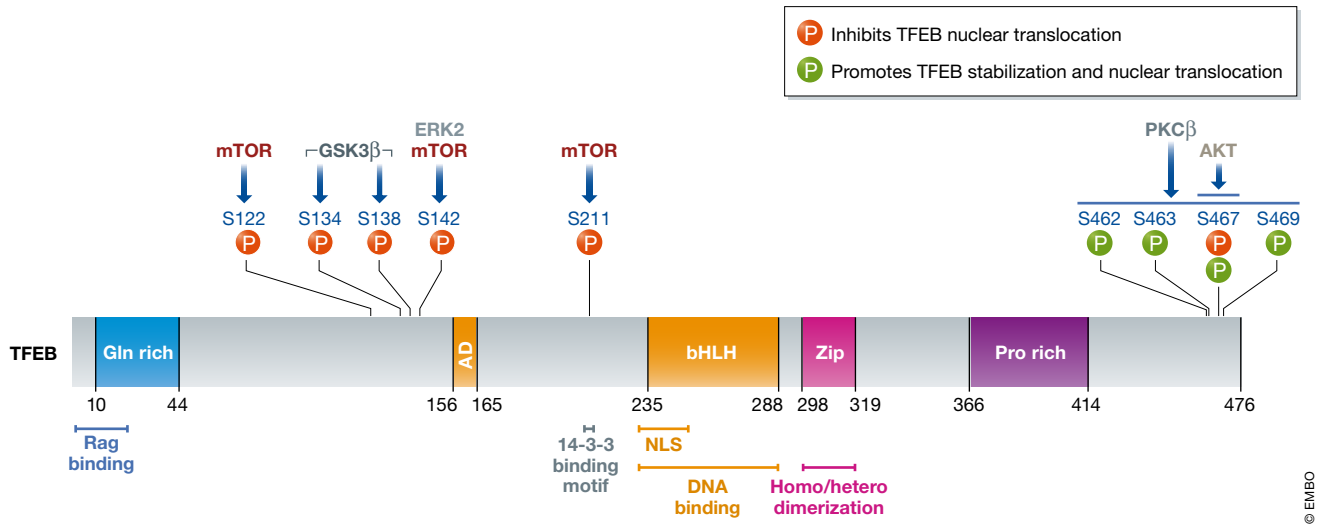

Figure 2. Relevant TFEB phosphorylation sites and their regulatory role.

mechanisms are conserved between TFEB, TFE3, and MITF (Martina *et al*, 2016, 2014; Wada *et al*, 2016; Fig 3).

Atypical regulation of TFEB by mTORC1 in TSC1/TSC2-deficient cells

While mTOR inhibition leads to TFEB nuclear localization and induction of lysosome biogenesis quite broadly across cell types, in some cellular contexts, this is not the case. In an unbiased screen to identify mTORC1-regulated genes, the expression of genes encoding v-ATPase subunits was upregulated in TSC1/TSC2-deficient cells, which retain mTORC1 activity under serum-starved conditions, but not in wild-type cells, in which mTORC1 was inhibited. Interestingly, v-ATPase expression in TSC1/TSC2-deficient cells could be downregulated by treatment with the mTORC1 inhibitor rapamycin (Pena-Llopis *et al*, 2011). v-ATPase expression in TSC1/TSC2-deficient cells was associated with nuclear TFEB and was dependent on it. Specifically, TFEB depletion using shRNA led to the downregulation of v-ATPases. These data suggest that mTORC1 promotes TFEB nuclear localization and expression of target genes such as v-ATPases in TSC1/TSC2-deficient cells. Consistent with this notion, siRNA-mediated silencing of the mTORC1 essential subunit Raptor in these cells excluded TFEB from the nucleus and led to a reduction in v-ATPase expression. Notably, when a C-terminal serine-rich motif (S462/463/466/467/469) was mutated to aspartate, TFEB remained constitutively nuclear. Furthermore, $TFEB^{5xSD}$ localization was unaltered by rapamycin. Why mTORC1 promotes rather than inhibits TFEB in TSC1/TSC2-deficient cells remains unclear. The data show, however, that TFEB regulation by mTORC1 is plastic and cell context dependent.

Intriguingly, the subcellular localization of TFE3, whose regulation by mTOR is similar to TFEB, appears to be independent from both Rheb and TSC2 activities in adipocytes (Wada *et al*, 2016). In addition, while the mTORC1/mTORC2 inhibitor Torin 1 inhibits TFEB phosphorylation at low doses, the specific mTORC1 inhibitor rapamycin did not affect TFEB phosphorylation and subcellular localization even at high doses (Settembre *et al*, 2012; Kang *et al*, 2013). These data suggest that TFEB may be an atypical mTORC1 substrate and that mTORC1 regulation of TFEB is more complex than generally appreciated and can be affected by cellular context.

Other kinases

In addition to mTORC1, other kinases were found to phosphorylate TFEB. In osteoclasts, PKCβ phosphorylates multiple serine residues (i.e., S462, S463, S467, and S469) located in the C-terminus of human TFEB. Phosphorylation of these serines by PKCβ is important for TFEB protein stability but does not affect TFEB subcellular localization (Ferron *et al*, 2013). ERK2 was also found to phosphorylate TFEB. Phosphorylation of TFEB S142 by ERK2 promotes TFEB cytoplasmic retention. Accordingly, treatment with ERK inhibitors and silencing of ERK2 resulted in TFEB nuclear translocation (Settembre *et al*, 2011). The relationship between mTORC1-mediated and ERK2-mediated phosphorylation of TFEB S142 is still unclear. However, ERK-mediated phosphorylation of the homologous S73 site in MITF was reported to control transcriptional activity and protein stability (Wu *et al*, 2000).

Transcription factor EB can be phosphorylated by GSK3 at residues S134 and S138 leading to cytoplasmic retention, whereas GSK3 inhibition led to TFEB nuclear translocation (Li *et al*, 2016). The phosphorylation of these serines by GSK3 mediates the recruitment of TFEB to the lysosome by an unknown mechanism. S134A and S138A mutations impair lysosomal recruitment of TFEB, thus indirectly impairing mTORC1-mediated phosphorylation. Based on these observations, the effects of GSK3 on TFEB may not be truly independent from mTORC1. Similar GSK3-dependent phosphorylation on evolutionarily conserved sites in MITF resulted in stabilization and enhanced function (Ploper *et al*, 2015).

A recent study showed that TFEB is phosphorylated by AKT at serine residue S467 and that treating cells with an AKT inhibitor promotes TFEB nuclear translocation (Palmieri *et al*, 2017). In addition, trehalose, a known autophagy activator, was shown to inhibit Akt activity, thus promoting TFEB nuclear translocation. A mutant form of TFEB carrying an S467A mutation shows an increased nuclear localization in normally fed cells compared to wild-type

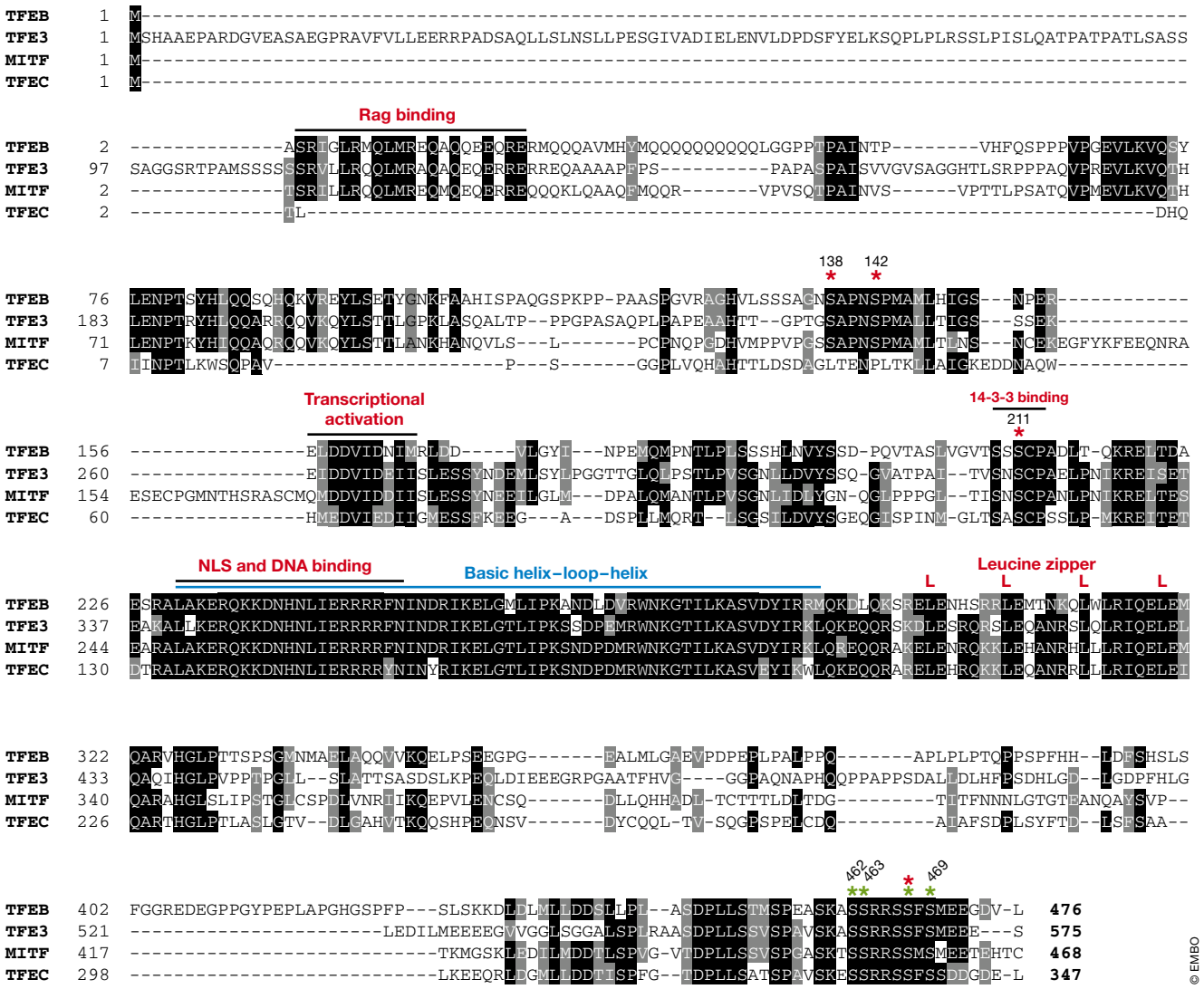

Figure 3. Sequence conservation of TFEB, TFE3, MITF and TFEC phosphorylation sites.
Multiple sequence alignment highlights the conservation of critical domains and phosphorylation sites between human TFEB (Uniprot P19484), TFE3 (Uniprot 19532), MITF (O75030, "D" splice variant shown here), and TFEC (Uniprot O14948) proteins. Red asterisks highlight phosphorylation sites that inhibit nuclear translocation of TFEB, while those shown in green have been found to promote TFEB nuclear localization.

TFEB. This effect appears to be independent from mTOR-mediated TFEB phosphorylation. However, the effects of AKT inhibition on mTORC1 activity were not tested, nor was the effect of the S467A mutation on TFEB serine S122, S211, or S142 phosphorylation. Thus, also in this case, a dependence on the mTOR pathway cannot be formally excluded (Palmieri *et al*, 2017).

Finally, another recent study showed that the curcumin analogue C1 promotes TFEB nuclear translocation in a phosphorylation-independent way. In this case, C1 binds directly to TFEB, manner that interferes with binding of TFEB to 14-3-3 proteins (Song *et al*, 2016).

De-phosphorylation of TFEB by calcineurin

As discussed above, to be active, TFEB needs to be de-phosphorylated, at least partially, at some key residues. Therefore, understanding how the process of TFEB de-phosphorylation occurs, and the phosphatases involved, is of critical importance to understand TFEB regulation. The search for the phosphatase responsible for TFEB de-phosphorylation was performed by siRNA-based high-content screening using an assay based on TFEB subcellular localization. Briefly, TFEB-GFP-expressing cells were starved in the presence of siRNAs directed against all the 231 known human phosphatases to identify the phosphatase(s) whose inhibition would prevent TFEB nuclear translocation.

This screening led to the identification of calcineurin as the phosphatase that plays a major role in TFEB de-phosphorylation (Medina *et al*, 2015). Interestingly, calcineurin was known to de-phosphorylate NFAT proteins, another family of transcription factors (Macian, 2005), and to promote their nuclear translocation. Inhibition of both calcineurin and mTORC1 activities results in TFEB cytoplasmic localization, indicating that the effects of

calcineurin inhibition override the effects of mTORC1 inhibition. These data suggest that calcineurin acts downstream of mTORC1 in the regulation of TFEB (Medina *et al*, 2015) or through a parallel pathway. Depletion or inhibition of calcineurin also causes a significant reduction in TFE3 activation in response to ER stress, revealing additional parallels in TFEB and TFE3 regulation (Martina *et al*, 2016).

Calcineurin is composed of a catalytic and a regulatory, calcium-dependent, subunit (Hogan & Li, 2005). This suggested that TFEB subcellular localization might be influenced by changes in intracellular calcium levels. Indeed, calcium chelators block starvation-induced TFEB nuclear translocation, while calcium ionophores promote TFEB nuclear translocation in a calcineurin-dependent manner (Medina *et al*, 2015). The lysosomal calcium channel mucolipin 1 (MCOLN1), also known as TRPML1, plays an important role in the activation of calcineurin and consequent TFEB de-phosphorylation (Medina *et al*, 2015; Zhang *et al*, 2016). Lysosomal calcium release via TRPML1 may increase calcium concentration near the lysosomal surface, and this may lead to local calcineurin activation. Thus, the lysosome acts as a calcium signaling hub by regulating TRPML1-Calcineurin-TFEB signaling. Interestingly, TRPML1 is also a direct transcriptional target of TFEB (Palmieri *et al*, 2011), suggesting the possibility of a positive feedback loop that involves TFEB and TRPML1.

Conclusions

Transcription factor EB and other members of the MiT-TFE family of transcription factors have emerged as important regulators of cellular energy metabolism. These transcription factors also appear to mediate communication between the lysosome and the nucleus in the adaptive response to environmental cues such as nutrient availability. Despite considerable progress made toward our understanding of how signaling pathways regulate TFEB subcellular localization and function via the phosphorylation of specific serine residues, several critical questions still remain unanswered. TFEB has been found to be phosphorylated by several kinases. However, the relationship and interdependence of these phosphorylation events are still unclear. It also remains to be established whether TFEB subcellular localization, in a physiological context, may be modulated by phosphorylation-independent mechanisms. Another important point to be addressed is how, once in the nucleus, TFEB is exported to the cytoplasm and whether TFEB nuclear export mechanisms add another layer of TFEB function regulation. Finding the answers to these and other critical questions will be of fundamental importance to our understanding of the transcriptional mechanisms that regulate cell metabolism in response to the environment and may lead to the development of powerful tools to modulate these pathways in human diseases.

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Conflict of interest

The authors declare that they have no conflict of interest.

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