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ANALYSIS OF THE TRANSCRIPTIONAL REGULATION OF mTORC1 ACTIVITY BY MIT/TFE TRANSCRIPTION FACTORS

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PUBLICATIONS


During my PhD, I was mainly involved in the project that was published on Science in 2017. I performed most of the *in vitro* experiments for the characterization of the MiT/TFE regulation of mTORC1, the characterization of the MiT/TFE-RagD-mTORC1 axis deregulation in cancers and some of the *in vivo* experiments.
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### LIST OF ABBREVIATIONS

- **4EBP**: eIF4E Binding Protein
- **ARF1**: ADP-ribosylation factor 1
- **ASPS**: Alveolar Soft Part Sarcoma
- **bHLH**: Basic helix-loop-helix domain
- **bHLH-LZ**: Basic helix–loop–helix leucine zipper
- **CLEAR**: Coordinated Lysosomal Expression and Regulation
- **DEPTOR**: DEP domain containing-mTOR interacting protein
- **E-box**: Enhancer box
- **EE**: Early endosome
- **ER**: Endoplasmic reticulum
- **ERK2**: Extracellular signal-regulated kinase 2
- **FLCN**: Folliculin
- **FKBP12**: FK506-binding protein
- **GAP**: GTPase activating protein
- **GEF**: Guanine nucleotide exchanging factor
- **HIF1α**: Hypoxia inducible factor 1α
- **IGF-1**: insulin/insulin-like growth factor-1
- **Lamtor**: Late endosomal/lysosomal adaptor, MAPK and MTOR activator
- **LE**: Late endosome
- **LRS**: Leucyl-tRNA Synthetase
- **LSDs**: Lysosomal Storage Disorders
- **MALAT1**: Metastasis associated lung adenocarcinoma transcript 1
- **MAPK1**: Mitogen-activated protein kinase 1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>MCLN1</td>
<td>Mucolipin 1</td>
</tr>
<tr>
<td>mLST8</td>
<td>Mammalian lethal with sec-13 protein 8</td>
</tr>
<tr>
<td>mSin</td>
<td>mammalian stress-activated map kinase-interactiprotein protein 1</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mechanistic target of rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mechanistic target of rapamycin Complex 1</td>
</tr>
<tr>
<td>mTORC2</td>
<td>Mechanistic target of rapamycin Complex 2</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localization Signal</td>
</tr>
<tr>
<td>PIKK</td>
<td>PI3K-related kinase family</td>
</tr>
<tr>
<td>PKCβ</td>
<td>Protein Kinase C beta</td>
</tr>
<tr>
<td>PRAS40</td>
<td>Proline-rich Akt substrate 40 kDa</td>
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<tr>
<td>Rag</td>
<td>Ras-related GTP binding protein</td>
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<tr>
<td>Protor1/2</td>
<td>Protein observed with rictor 1 and 2</td>
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<tr>
<td>Rag</td>
<td>Ras-related GTP binding protein</td>
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<tr>
<td>Raptor</td>
<td>Regulatory-associated protein of mammalian target of rapamycin</td>
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<tr>
<td>RCC</td>
<td>Renal Cell Carcinoma</td>
</tr>
<tr>
<td>Rictor</td>
<td>Rapamycin-insensitive companion of mTOR</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal Pigmented Epithelium</td>
</tr>
<tr>
<td>S6K1</td>
<td>p70S6 Kinase 1</td>
</tr>
<tr>
<td>SREBP1/2</td>
<td>sterol regulatory element-binding protein 1/2</td>
</tr>
<tr>
<td>TFE3</td>
<td>Transcription Factor E3</td>
</tr>
<tr>
<td>TFEB</td>
<td>Transcription Factor EB</td>
</tr>
<tr>
<td>TFEC</td>
<td>Transcription Factor EC</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous Sclerosis Complex</td>
</tr>
<tr>
<td>TSS</td>
<td>Transcription Start Site</td>
</tr>
<tr>
<td>ULK1</td>
<td>Unc-51 like autophagy activating kinase 1</td>
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</table>
**UPS**  Ubiquitin-proteasome system

**v-ATPase**  Vacuolar H⁺-ATPase
ABSTRACT

The mechanistic Target Of Rapamycin Complex 1 (mTORC1) regulates cellular biosynthetic pathways in response to variations in nutrient availability. Activation of mTORC1 is mediated by Rag GTPases, that act as heterodimers and promote mTORC1 recruitment to the lysosome. Many studies have clarified the post-translational control of mTORC1, but little is known about its transcriptional regulation. Our study demonstrates that TFEB, TFE3 and MITF, members of the MiT/TFE family of transcription factors and master regulators of lysosomal and melanosomal biogenesis and autophagy, are nutrient-sensitive transcriptional activators of mTORC1 signaling. During starvation they induce the expression of the RagD gene and this enhances mTORC1 recruitment to the lysosome and its reactivation when nutrients become available. Thus, in periods of nutrient deprivation, this mechanism allows the cell to rapidly reactivate anabolic pathways and turn off catabolism when nutrient levels are restored. Furthermore this mechanism plays an important role in cancer growth. Up-regulation of the MiT/TFE genes in renal cell carcinoma and melanoma is associated to RagD-induced mTORC1 activation, causing cell hyperproliferation and cancer progression.
INTRODUCTION

CHAPTER 1. MiT/TFE transcription factors

1.1 MiT/TFE transcription factors family.

Over the years, MiT/TFE factors have emerged as key players in several basic cellular processes. The family is composed of 4 members: TFEC, TFEB, TFE3 and MITF and it is considered a subgroup of basic Helix-Loop-Helix Leucine Zipper (b-HLH-LZ) transcription factors (Hemesath et al., 1994). In mammals, most of the b-HLH-LZ factors are involved in cell proliferation and developmental processes and they usually bind to an hexameric sequence, CACGTG, called E-box. Moreover, the MiT/TFE factors are also able to bind to M-box (CATGTG) and to the non canonical E-box (TCATGTG or TCATGTGA) (Hemesath et al., 1994; Aksan and Godin, 1998). These factors share three conserved domains: the DNA-binding region, that recognize specific target sequences, and the HLH and LZ regions, required for their protein-protein interactions (Steingrímsson et al., 2004). Homo or hetero-dimerization is required for the binding to specific DNA sequences (Hemesath et al., 1994) and MiT factors are able to dimerize only among themselves and not with other b-HLH-LZ factors (Hemesath et al., 1994; Pogenberg et al., 2012). The functional relevance of these homo/hetero-dimerizations is still unknown.

TFEB, TFE3 and MITF also present a conserved activation domain, important for their role as transcriptional activators, which is missing in TFEC (Beckmann et al., 1990; Sato et al., 1997; Steingrímsson et al., 2004). In fact, TFEC is reported to share less common features with the other members, in terms of sequence and also in functions (Zhao et al., 1993) and its expression is restricted to
monocyte/macrophage lineage (Rehli et al., 1999). A recent work shows its involvement in Hematopoietic Stem Cell expansion in zebrafish, but its role is still largely unknown (Mahony et al., 2016). MITF factor is mostly expressed in melanocytes, but it is described to have prominent roles also in mast-cells, osteoclasts and in the retinal pigmented epithelium (RPE) (Steingrímsson et al., 2004). Melanocytes, together with the RPE, are responsible for skin, hair and eye colour. In these cells, MITF is a master regulator of melanosomal differentiation, development and survival and play a crucial role in the generation of action potentials in the inner ear (Hodgkinson et al., 1993; Hemesath et al., 1994). Mice lacking of a functional MITF gene show white coat, due to the absence of pigmentation, and eye developmental defects, as a consequence of MITF absence in the RPE (Steingrímsson et al., 2004). In humans, MITF mutations are associated to the Waardenbrug Syndrome type 2, an inherited disorder associated with hearing loss and pigmentation defects (Tassabehji et al., 1994).

Less than a decade ago, TFEB was identified as a master regulator of lysosomal and autophagosomal genes (Sardiello et al., 2009; Settembre et al., 2011). Following studies demonstrated that also TFE3 and some isoforms of MITF were able to bind to the same consensus sequence named Coordinated Lysosomal Expression and Regulation (CLEAR) (Martina et al., 2014). TFEB and TFE3 are ubiquitously expressed and studies in mice indicated that they share similar functions, such as involvement in humoral immunity (Huan et al., 2006) and the cooperation in the regulation of glucose and lipid metabolism (Pastore et al., 2017). These evidences suggest that TFEB and TFE3 are redundant factors, but their roles are not completely overlapping, since Tcfeb Knock-Out (KO) mice are not viable, due to defects in placental vascularization (Steingrímsson et al., 1998),
while Tfe3 KO mice showed no embryonic developmental defects (Steingrímsson et al., 2002).

1.2. The lysosomes

Christian de Duve in the early 50's discovered the lysosomes, intracellular vesicles that he named with a Greek word meaning "digestive bodies" (de Duve, 2005). These cellular organelles are considered the "trash can" of the cells, since they degrade and recycle cellular waste. Since their discovery, several studies have revealed that the lysosome is much more than this, and it is a real cellular metabolic hub with multiple different roles as nutrient sensing, gene regulation, secretion, plasma membrane repair, metal ion homeostasis, cholesterol transport, and immune response (Safting and Klumperman, 2009; Lim and Zoncu, 2016; Perera and Zoncu, 2016). In particular, the main lysosomal functions can be grouped in degradation, secretion and signaling.

1.2.1 Lysosomal degradation

Lysosomes are primarily known as the cell's degradation center. They receive most of the extracellular material via endocytosis or phagocytosis, while intracellular material is mainly delivered by autophagy (Luzio et al., 2007). Endocytosis or phagocytosis start from the plasma membrane with the formation of endocytic vesicles that subsequently pass through a maturation process: from Early Endosomes (EEs) they become Late Endosomes (LEs) and all the compartments formed during these maturation process differ in components and functions. The process ends when LEs finally fuse with lysosomes, forming endolysosomes, and, at this stage, all the materials transported in this newly formed
compartment is degraded (Xu and Ren, 2015). On the contrary, during the autophagy process there are no vescicular maturation steps, but the materials are directly delivered to the lysosomes. Usually, the terms "autophagy" is referred to the so called "Macroautophagy": double-membrane vescicles, called autophagosomes, engulf damaged organelles, long lived proteins and pathogens, and they fuse directly with lysosomes, degrading all the cargos transported (Settembre et al., 2013b). The breakdown of all the molecules received (proteins, polysaccharids, complex lipids,...) into their building-blocks (amino acids, monosaccharids, free fatty acids,...) is accomplished by about 60 different hydrolases (lipases, proteases, glycosidases,...) that fill the lysosomal lumen (Xu and Ren, 2015). The presence, on the lysosomal membrane, of the vacuolar H$^+\,$-ATPases complex (v-ATPase), together with other ion channels, allow to constantly pump protons (H$^+$) into the lumen, adjusting the pH to ~4.5-5 (Lim and Zoncu, 2016). This acidification is required for lysosomal hydrolitic activities. The proton gradient is also the driving force that directs newly synthesized hydrolitic enzymes from Golgi to lysosomes and allows the recycling of the cargos back to the Golgi (Settembre et al., 2013b).

1.2.2 Lysosomal secretion

The lysosomal membrane is enriched in ion channels that transport ions like Na$^+$, K$^+$, Ca$^{2+}$, Cl$^-$. This ion movement across the lysosomal membrane generate a membrane potential that helps regulating the v-ATPase proton gradient and it is crucial for the process of lysosomal secretion, more often called "lysosomal exocytosis" (Perera and Zoncu, 2016). The lysosomes dock to the cell surface and the increasing levels of Ca$^{2+}$ induce their fusion with the plasma membrane,
secreting their content into the extracellular compartment (Vernhage and Toonen, 2007). Despite the initial hypothesis of the exocytosis to occur only in some type of secretory cells, this process was then observed in all the cell types (Rodríguez et al., 1997). Lysosomal exocytosis mediates several physiological processes, such as degranulation in cytotoxic T lymphocytes, bone resorption by osteoclasts, parasite defence by mast cells and eosinophils, melanocyte function in pigmentation, platelet function in coagulation and hydrolase release by spermatozoa during fertilization (Settembre et al., 2013b). Moreover, lysosomal exocytosis has also an important role in repairing plasma membrane damages, since these injuries recall lysosomes to the involved site which then fuse with the plasma membrane, repairing the disrupted sites (Reddy et al., 2001).

1.2.3 Lysosomal signaling

Over the years, it has become evident that the lysosomes are not simply the "trash can" of the cells, but they are involved in maintaining cellular metabolic homeostasis. Cells are able to adapt to nutrient changes and regulate accordingly cell growth and metabolism mainly thanks to mechanistic Target of Rapamycin Complex 1 (mTORC1) signaling pathway (Laplante and Sabatini, 2012; Saxton and Sabatini, 2017). Moreover, a crosstalk exists between the nutritional status of the cell, sensed by mTORC1, which function on the lysosomal surface, and TFEB, which transfers the information received to the nucleus (Settembre et al., 2012).
1.3 Transcription Factor EB (TFEB): a master regulator of lysosomal function and autophagy

Originally, lysosomes were described as static organelles, involved only in degrading and recycling cellular waste, while further studies showed their involvement in the regulation of several processes important for the maintenance of cellular homeostasis (Lim and Zoncu, 2016; Settembre et al., 2013b). In 2009 analysis of microarray data revealed that lysosomal genes are co-expressed in different cell types and conditions, suggesting the presence of one or more regulators (Sardiello et al., 2009). Promoter analysis of lysosomal genes resulted in the identification of a palindromic sequence of 10 base-pairs (GTCACGTGAC) localized mainly within 200 base-pairs from the transcription start site (TSS), the CLEAR element, which contained the consensus E-box (CANNTG) that is typically recognized by bHLH-LZ transcription factors (Sardiello et al, 2009). TFEB directly binds to CLEAR elements and induce the expression of its target genes, such as several subunits of the v-ATPase, lysosomal hydrolases and lysosomal transmembrane proteins (Sardiello et al, 2009; Palmieri et al., 2011). Furthermore, TFEB overexpression increases the number of lysosomes and the levels of lysosomal enzymes, boosting their catabolic function (Sardiello et al, 2009; Palmieri et al., 2011). Subsequently, further studies revealed that TFEB regulates genes not only involved in lysosomal biogenesis and function, but also in autophagy and lysosomal exocytosis (Palmieri et al., 2011). Specifically, TFEB binds to promoters of genes that regulates autophagosome biogenesis and autophagosome-lysosome fusion and can promote the degradation of autophagy substrates, like long-lived proteins (Settembre et al., 2011). Moreover, TFEB induces the clearance of lipid droplets and damaged mitochondria (Settembre et
TFEB has also been found to regulate lysosomal exocytosis, increasing the release of intracellular Ca\textsuperscript{2+} and the number of lysosomes ready to fuse with the plasma membrane (Medina et al., 2011). Thus TFEB coordinates a complex transcriptional program that regulates the main degradative pathways and promotes cellular clearance. Moreover, it has been showed that TFEB overexpression can ameliorate the phenotype of mouse models with pathological conditions associated to lysosomal disfunction, such as Lysosomal Storage Disorders (LSDs), but also more common diseases like Parkinson's, Alzheimer's, Huntington's (Settembre et al., 2013a; Ballabio, 2016).

### 1.4 TFEB regulation

The identification of TFEB as the "master regulator of lysosomes" supports the idea of these organelles as signaling platforms. TFEB regulation is dependent on post-translational modifications, subcellular localization and protein-protein interactions. In basal condition, when nutrients are present, TFEB is inactive into the cytosol, whereas, in starvation condition, when nutrients are no longer available, or in case of lysosomal stress, TFEB translocate into the nucleus and activates its target genes (Settembre et al., 2011; Martina et al., 2012; Roczniak-Ferguson et al., 2012). TFEB subcellular localization is mostly regulated by the phosphorylation of two critical serine residues, Ser142 (Settembre et al., 2011; 2012) and Ser211 (Martina et al., 2012; Roczniak-Ferguson et al., 2012; Settembre et al., 2012): when these serines are phosphorilated TFEB is retained into the cytosol. ERK2 (or MAPK1) and mTORC1, both involved in the regulation of cellular growth, are the main kinases that phosphorylate TFEB respectively on Ser142 and on both Ser142 and Ser211. Mutations of these serines into alanines result in a constitutive nuclear TFEB, always active (Martina et al., 2012;
Rocznia-Ferguson et al., 2012; Settembre et al., 2011, 2012). In osteoclasts, another kinase, protein kinase Cβ (PKCβ), phosphorylates TFEB on serine residues different from the previous ones: this event stabilizes the transcription factor, inducing lysosomal biogenesis and bone resorption in osteoclasts (Ferron et al., 2013). Despite all the residues phosphorylated by these kinases, the phosphorylation of Ser211 by mTORC1 results to be the key residue responsible for TFEB cytosolic sequestration, since it promotes the binding of TFEB to the chaperone 14-3-3. Thus TFEB is unable to translocate into the nucleus, probably because the binding with this chaperone masks its Nuclear Localization Signal (NLS) (Martina et al., 2012; Rocznia-Ferguson et al., 2012). Furthermore, both mTORC1 and ERK2 exert their activity on the lysosomal surface (Sancak et al., 2010; Nada et al., 2009).

Once understood that TFEB cytosolic localization is mainly regulated by phosphorylation events, the following question was: what is the phosphatase involved in its dephosphorylation? Later studies demonstrated that nutrient deprivation induces the release of lysosomal Ca^{2+} through the Ca^{2+} channel mucolipin 1 (MCOLN1); this activates the phosphatase calcineurin, which in turn dephosphorylates TFEB and promotes its nuclear translocation (Medina et al., 2015). MCOLN1 is crucial for lysosomal Ca^{2+} release and calcineurin activation, since depletion of MCOLN1 inhibits these processes, thus preventing TFEB activation (Medina et al., 2015) (Fig.1)
Fig.1. Model of TFEB regulation.

Under normal feeding conditions, TFEB is phosphorylated by mTORC1 on the lysosomal surface and is sequestered in the cytoplasm by the 14-3-3 proteins. During starvation, Ca^{2+} is released from the lysosome through MCOLN1 and this leads to local calcineurin (Cn) activation and TFEB dephosphorylation. Dephosphorylated TFEB is no longer able to bind 14-3-3 proteins and can translocate to the nucleus where it transcriptionally activates the lysosomal/autophagic pathway.

Interestingly, TFEB regulates the transcription of many genes important for its own regulation, such as MCOLN1 and genes encoding for v-ATPase subunits and numerous lysosomal enzymes. This supports the idea that lysosomal adaptation to external stimuli is regulated by multiple feedback loops. In accordance with this, TFEB induces its own transcription, in a positive autoregulatory loop that is dependent on the presence of CLEAR elements located in its promoter (Settembre et al., 2013a).
The same mechanisms of regulation are conserved for the other MiT/TFE factors. In particular, both TFE3 and some isoforms of MITF are phosphorylated by mTORC1 (Martina and Puertollano, 2013; Martina et al., 2014).

### 1.5 MiT/TFE factors in cancer

MiT/TFE are known oncogenes deregulated in different cancer types (Haq and Fisher, 2011). Below their involvement in Renal Cell Carcinoma and Melanoma is described.

#### 1.5.1 Renal Cell Carcinoma

Renal Cell Carcinoma (RCC) is the most common form of kidney cancer and includes different hystopathological subtypes (Linehan et al., 2010). Approximately 5% of sporadic RCC tumors is part of a rare subgroup named translocation-RCC (t-RCC), most common in children and characterized by translocations and rearrangements involving TFE3, and less commonly TFEB genes (Kauffman et al., 2014). T-RCC associated TFE3 gene fusions can occur with several partners including PRCC, ASPSCR1, SFPQ, NONO and CLTC (Kauffman et al., 2014; Linehan et al., 2010). ASPSCR1–TFE3 and SFPQ–TFE3 fusions are not restricted to RCC, being originally identified in Alveolar Soft Part Sarcoma (ASPS) and in a subset of benign tumors, known as perivascular epithelioid cell neoplasms (Argani et al. 2001; Tanaka et al., 2009). Regarding the fusion proteins obtained, rearrangements can involve different introns of the partner genes, that can vary in patients: this results in different mRNA isoforms, thus different fusion proteins in size. However, the fusion proteins always retain a 280 amino acid C-terminal
portion harboring the bHLH–LZ dimerization, DNA-binding domain and the putative NLS of TFE3 (Kauffman et al., 2014). In the case of TFEB, a 6p21/11q13 translocation results in the fusion of the TFEB coding region with the regulatory region of the non-coding MALAT1 gene (Davis et al., 2003). Usually the MALAT1-TFEB breakpoints are located before the start codon in exon 3 of the TFEB coding sequence, thus resulting in the translation of the full length TFEB protein under the control of the strong MALAT1 promoter (Inamura et al., 2012). TFEB-tRCCs patients usually have a better prognosis than TFE3-tRCC patients. More recently, a comprehensive genomic analysis of 161 primary papillary RCC led to identification of novel fusion partners for both TFE3 and TFEB, including RBM10 and DVL2 for TFE3 and COL21A1 and CADM2 for TFEB (Cancer Genome Atlas Research Network et al. 2016). The mechanisms explaining the oncogenic role of the TFE-fusion proteins in RCC are still unclear. Several hypothesis came out during the years, one is that these TFE-fusion proteins retain partially the functions of the partner genes, oftenly involved in promoting tumorigenesis (NONO, SFPQ, PRCC appear to have regulatory roles in mRNA splicing, whereas PRCC, CLTC are implicated in mitosis) (Kauffman et al., 2014). Another hypothesis is that the fusion leads to upregulation of oncogenic activity already present in the wild-type protein: this is the case of TFEB-MALAT1 gene (Kuiper et al., 2003), but also TFE3 fusion proteins show much higher expression levels than the wild-type TFE3 (Argani et al., 2003; Clark et al.,1997; Weterman et al., 1996; 2000). No MITF-fusion proteins have been associated to RCC.

1.5.3 Melanoma
Melanoma is an aggressive type of cancer involving the melanocytes. The most common type is the cutaneous melanoma, arising from the epidermal melanocytes
of the skin. Approximately 50% of melanoma are characterized by V600E activating mutation in the BRAF kinase, resulting in hyper-activation of the MAPK signaling pathway (Davies et al., 2002; Hodis et al., 2012). Amplification of the MITF gene was detected in 5-20% of melanoma and these tumors appear to have better prognosis (Genomic Classification of Cutaneous Melanoma 2015). MITF-associated tumors are usually caused by gene amplifications, but somatic mutations of MITF have also been reported. These mutations are generally located in the MITF transactivation domain, indicating a role of the transcriptional activity of MITF in driving tumorigenesis (Cronin et al., 2009). Several studies claim that MITF levels influence different mechanisms and drive the response to therapies (Ennen et al. 2015; Hoek et al. 2008; Tirosh et al. 2016).
CHAPTER 2. The mechanistic Target of Rapamycin Complex 1 (mTORC1)

2.1 The mechanistic Target of Rapamycin (mTOR) complexes

Originally, the mechanistic Target of Rapamycin (mTOR) was identified as the target of Rapamycin in mammals, a compound that inhibits cell growth and proliferation (Sabatini et al., 1994). mTOR is a serine/threonine kinase in the PI3K-related kinase family (PIKK), implicated in the catalytic activity of two different complexes: mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2). The complexes differ in their subunits and in their function. As for their composition, both mTOR complexes are quite big, with mTORC1 having six and mTORC2 seven known protein components. They share the catalytic mTOR subunit, and also mammalian lethal with sec-13 protein 8 (mLST8, also known as GβL) (Jacinto et al., 2004; Kim et al., 2003), which interacts with the catalytic domain of mTOR and stabilize the kinase activation loop (Yang et al., 2013), DEP domain containing mTOR-interacting protein (DEPTOR), an inhibitor subunit (Peterson et al., 2009), and the Tti1/Tel2 complex, positive regulators of the complexes (Kaizuka et al., 2010). However, two other subunits are specific for mTORC1: regulatory-associated protein of mammalian target of rapamycin (raptor), required for proper mTORC1 subcellular localization and substrates recruitment (Kim et al., 2002) and proline-rich Akt substrate 40 kDa (PRAS40), an inhibitor subunit (Sancak et al., 2007). On the contrary, mTORC2 is composed by three additional subunits: rapamycin-insensitive companion of mTOR (riCTOR), with analogous function of raptor (Sarbassov et al., 2004), and the two regulatory subunits mammalian stress-activated map kinase-interacting protein 1 (mSin1) (Jacinto et al., 2006) and
protein observed with rictor 1 and 2 (protor1/2) (Pearce et al., 2007). The two complexes have different functions. mTORC1 responds to amino acids, stress, oxygen, energy, and growth factors, promoting cell growth and cell-cycle-progression and is acutely sensitive to rapamycin (Laplante and Sabatini, 2012). In fact, rapamycin forms a complex with the intracellular 12-kDa FK506-binding protein (FKBP12) (Sabatini et al., 1994), masking mTOR catalytic domain and thus blocking the phosphorylation of its substrates (Yang et al., 2013). Rapamycin-FKBP12 complex directly interacts with and inhibits mTOR when it is part of mTORC1 but not mTORC2 (Yang et al., 2013). mTORC2 responds to growth factors and regulates cell survival and metabolism, as well as the cytoskeleton, and it is insensitive to acute rapamycin treatment (Laplante and Sabatini, 2012). Actually, prolonged exposure to this drug abolish mTORC2 signaling, probably due to the absence of unbound mTOR available to form new complexes (Sarbassov et al., 2006).

2.2 Upstream regulators of mTORC1

Nutrient availability is a major determinant of survival and growth and most of the species have evolved mechanisms to efficiently switch between anabolic and catabolic states in order to deal with its fluctuation in the environment. mTORC1 is a main regulator of the metabolic adaptation to nutrient availability: it is activated in feeding conditions, to promote growth and energy storage, whereas the complex activity is inhibited in fasting conditions, to avoid the waste of insufficient nutrients (Laplante and Sabatini, 2012; Saxton and Sabatini, 2017). Different stimuli can exert their effects on mTORC1 pathway, by modulating the activity of the components of this complex. Several growth factors, such as the insulin/insulin-like growth factor-1 (IGF-1) pathway (Inoki et al., 2002), and
different mitogen-dependent signaling pathways, like the receptor tyrosine kinase-dependent Ras signaling (Roux et al, 2004), converge in the activation of mTORC1 signaling, through the inhibition of the Tuberous Sclerosis Complex (TSC), an heterotrimeric complex, composed by the subunits TSC1, TSC2 and TBC1D7 (Dibble et al, 2012). TSC is a crucial negative regulator of mTORC1, acting as a GTPase activating protein (GAP) for Rheb, a small GTPase, which resides at the lysosomal surface and activate mTORC1 when it is bound to GTP (Inoki et al., 2003; Long et al., 2005). TSC2 inhibition via Akt-dependent inhibitory phosphorylation, causes the dissociation of the TSC complex from the lysosomes hence removing Rheb inhibition and allowing mTORC1 activation (Inoki et al., 2002; Roux et al., 2004).

Other stimuli associated to cellular stress modulate mTORC1 activity, such as hypoxia, low ATP levels, glucose deprivation and DNA damage (Saxton and Sabatini, 2017). Most of these stimuli inhibit mTORC1 activity inducing the stress responsive metabolic regulator AMPK, which block mTORC1 signaling both indirectly, by TSC2 activating phosphorylation, and directly, phosphorylating raptor and causing an allosteric inhibition of mTORC1 (Gwin et al., 2008).

2.2.1 Amino acid sensing by mTORC1

Amino acids levels are crucial for mTORC1 activity and this is one of the most conserved growth signal to mTORC1 pathway. After decades of studies, amino acid sensing by mTORC1 is still not fully understood. As example, it is still not completely clear if all the 20 amino acids have the same contribution to the signaling. Leucine and arginine are fundamental for mTORC1 activation but they are not sufficient if the other 18 amino acids are absent in the cell (Hara et al., 1998). Amino acids directly regulate mTORC1 recruitment to the lysosome by
modulating the GTP/GDP bound state of the RagGTPases (Kim et al., 2008; Sancak et al., 2008). Mammals have four RagGTPases (Rags): RagA, RagB, RagC and RagD. They function as obligate heterodimers composed of RagA or RagB, together with RagC or RagD. When amino acids are available, the heterodimer is composed by the Rags in their active state: RagA/B-GTP bound and RagC/D-GDP bound, and it localizes to the lysosome. This GTP/GDP bound state is responsible for Rags interaction with raptor and thus mTORC1 lysosomal localization, where the complex can be activated by Rheb (Sancak et al., 2008; Inoki et al., 2003; Long et al., 2005). Rags are anchored to the lysosomal surface through a pentameric complex, called Ragulator (Sancak et al., 2010). Ragulator was identified as a Rag-interacting complex and its basic architecture consists of Lamtor1 (late endosomal/lysosomal adaptor, MAPK and MTOR activator 1) which represents a scaffold for two obligate heterodimers composed of Lamtor2–Lamtor3 and Lamtor4–Lamtor5 (Sancak et al., 2010). Post-translational modifications, such as myristoylation and palmitoylation on the N terminus of Lamtor1, promote the localization of Ragulator and Rag GTPases to lipid rafts on lysosomal surface (Nada et al., 2009). Moreover, initial studies indicated that Ragulator is a guanine nucleotide exchanging factor (GEF) for RagA/B which binds to RagA/B in their inactive state (GDP-bound), to promote the switch from GDP to GTP (Bar-Peled et al., 2012). However, very recently new data have emerged indicating that even if Ragulator is implicated in RagA/B activation, their real GEF is the amino acid sensor SLC38A9 (Shen and Sabatini, 2018). However, the role of RagC/D is equally important, they have to be loaded with GDP to favour Rags’ heterodimer interaction with Raptor, allowing translocation of mTORC1 to the lysosome (Tsun et al., 2013). Folliculin (FLCN), together with its partner FNIP1/2, acts as GTPase Activating Protein (GAP) for RagC/D, promoting the
hydrolysis of GTP to GDP (Tsun et al., 2013). In addition, GATOR complexes are key players of mTORC1 sensing machinery. GATOR is an octameric complex, composed by two subcomplexes called GATOR1 and GATOR2, respectively having 3 and 5 subunits: GATOR1 negatively regulates mTORC1 signaling, functioning as a GAP for RagA/B, whereas GATOR2 inhibits GATOR1 (Bar-Peled et al., 2013).

MTORC1 can sense both intra-lysosomal and cytosolic amino acids through different mechanisms. Inside the lysosomal lumen, amino acids are able to regulate the nucleotide state of the Rags through the lysosomal v-ATPase, which, somehow, communicates amino acids abundance to the activating complex for RagA/B composed by Ragulator and SLC38A9 (Zoncu et al., 2011, Shen and Sabatini, 2018). SLC38A9 is a transmembrane protein, which also acts as sensor for arginine (Wang et al., 2015). Some other amino acid sensors have also been recently found. Sestrin2 is a GATOR2 interacting protein, working as leucine sensor for mTORC1, that binds and inhibits GATOR2 in absence of leucine, or it dissociates from GATOR2 in presence of leucine, thus relieving GATOR1 inhibition of mTORC1 (Chantranupong et al., 2014; Wolfson et al., 2016).

Cytosolic arginine is instead communicated to mTORC1 signaling through a different sensor, CASTOR1, which associate or dissociate from GATOR2 in absence or in presence of arginine, respectively, resulting in mTORC1 activation only when arginine is available (Chantranupong et al., 2016). Finally, glutamine, the most abundant free amino acid in the cell, seems to be able to promote mTORC1 lysosomal recruitment independently from Ragulator and Rags, via a mechanism that appears to involve the v-ATPase and ADP-ribosylation factor 1 (ARF1) (Jewell et al., 2015).
Hence, amino acids represent a crucial stimuli that regulates mTORC1 activity, and further studies will be required to fully dissect all the components of this complex sensing machinery (Fig.2).

Fig.2. The nutrient sensing pathway upstream of mammalian mTORC1.
Schematic model of the main components involved in amino acids regulation of mTORC1 activity.

2.3 Downstream of mTORC1

In order to promote cellular growth and proliferation, mTORC1 senses nutrients availability and controls the balance between anabolism and catabolism. Thus, mTORC1 activates protein, lipid and nucleotide synthesis when nutrients are available, while inhibiting catabolic processes, like autophagy and lysosome biogenesis (Laplante and Sabatini, 2012; Saxton and Sabatini, 2017).
2.3.1 Anabolic processes

MTORC1 promotes protein synthesis mainly through the phosphorylation of two key effectors, p70S6 Kinase 1 (S6K1) and eIF4E Binding Protein (4EBP). MTORC1 directly phosphorylates S6K1 at the Thr389, enabling its subsequent phosphorylation and activation by PDK1. As a consequence, S6K1 phosphorylates and activates several substrates that promote mRNA translation initiation, such as eIF4B, a positive regulator of the 5' cap binding eIF4F complex (Holz et al., 2005). S6K1 also phosphorylates and promotes the degradation of PDCD4, an inhibitor of eIF4B (Dorrello et al., 2006), and enhances the translation efficiency of spliced mRNAs through its interaction with SKAR, a component of exon-junction complexes (Ma et al., 2008). Moreover, S6K1, together with its substrate ribosomal protein S6 (S6), was also thought to control the translation of an abundant subclass of mRNAs characterized by an oligo-pyrimidine tract at the 5' end (5' TOP mRNAs) and that encode most of the protein components of the translational machinery. Nevertheless, even if mTORC1 is a key regulator of the translation of these mRNAs, S6K1 and S6 are not involved in this process (Tang et al., 2001). The substrate 4EBP is another mTORC1 direct substrate and inhibits translation by binding and sequestering eIF4E, blocking the assembly of the eIF4F complex. MTORC1 phosphorylates 4EBP at multiple sites, causing its dissociation from eIF4E, hence promoting the initiation of the cap-dependent translation (Gingras et al., 1999).

In addition to the regulation of proteins production, mTORC1 also controls the synthesis of lipids necessary for proliferating cells to generate new membranes. This function is mainly executed through the regulation of the sterol regulatory element-binding protein 1/2 (SREBP1/2) transcription factors that control the expression of numerous genes involved in fatty acid and cholesterol synthesis.
The inactive SREBP
ter on the endoplasmic reticulum (ER) and they are
activated in response to insulin or sterol depletion, translocating to the nucleus to
activate transcription of lipogenic genes (Du¨vel et al., 2010; Wang et al., 2011).
MTORC1 appears to regulate SREBP cellular localization (Wang et al., 2011).
Recent studies established that mTORC1 also promotes the synthesis of
cytotides required for DNA replication and ribosome biogenesis in growing and
proliferating cells. In fact, mTORC1 increases the ATF4-dependent expression of
MTHFD2, a key component of the mitochondrial tetrahydrofolate cycle involved in
purine synthesis (Ben-Sahra et al., 2016). Finally, mTORC1 positively regulates
chemical metabolism and ATP production: it increases glycolytic flux by activating
the transcription and the translation of hypoxia inducible factor 1α (HIF1α), a
positive regulator of many glycolytic genes (Brugarolas et al., 2003; Du¨vel et al.,
2010).

2.3.2 Catabolic processes
To promote cellular growth, in association with the induction of anabolic
processes, mTORC1 inhibits autophagy, the main degradative process in the cell.
An important early step in autophagy is the activation of ULK1, a kinase that forms
a complex with ATG13, FIP2000, and ATG101 and drives autophagosome for-
mation. In presence of nutrients, mTORC1 phosphorylates ULK1, thus preventing
its activation by AMPK, a key autophagy activator (Kim et al., 2011). In addition,
mTORC1 inhibits autophagy through the phosphorylation of TFEB, which results in
the downregulation of gene expression relative to its lysosomal and autophagy
targets. (Martina et al., 2012; Rocznik-Ferguson et al., 2012; Settembre et al.,
2012).
In addition to autophagy, a major pathway responsible for protein turnover is the ubiquitin-proteasome system (UPS), through which proteins are tagged with ubiquitin and selectively targeted for degradation by the 20S proteasome. Two recent studies found that acute mTORC1 inhibition rapidly increases proteasome activity through a general increase in protein ubiquitylation (Zhao et al., 2015) and an increase in the levels of proteasomal chaperones (Rousseau and Bertolotti, 2016). However, another study found that genetic hyperactivation of mTORC1 signaling also increases proteasome activity, through elevated expression of proteasome subunits downstream of Nrf1 (Zhang et al., 2014). One possible explanation for this discrepancy is that while acute mTORC1 inhibition promotes proteolysis to restore free amino acid pools, prolonged mTORC1 activation could cause a compensatory increase in protein turnover to balance the higher rate of protein synthesis. Thus, how exactly mTORC1 regulates this process is not yet clarified.

2.4 Physiological roles of mTORC1

Accordingly with the in vitro studies, mTORC1 regulates whole-body metabolism in mice, maintaining the perfect balance between in vivo anabolic and catabolic processes in response to nutrients.

During fasting, circulating glucose and amino acids levels rapidly decrease and autophagy, gluconeogenesis and the release of peculiar energy sources, such as ketone bodies, are promptly induced in the liver. In fact, mTORC1 seems to have a crucial role in regulating the response of the liver to diet. For example, liver conditional KO mice for tsc1, that are characterized by mTORC1 hyperactivation, are not able to generate ketone bodies, due to mTORC1 repression of PPARα, a
transcription factor that activates genes involved in ketogenesis (Sengupta et al., 2010). Another example of the importance of mTORC1 inhibition in regulating liver response to fasting is the phenotype showed by RagA\textsuperscript{GTP} mutant mice: constitutive activation of mTORC1 is associated with perinatal death, due to the inability of the newborns to maintain blood glucose homeostasis upon postnatal fasting (Efeyan et al., 2013). Indeed, mTORC1 plays an important role also in the regulation of glucose homeostasis: mice KO for tsc2 only in pancreatic β cells show, first, an increase in β cells mass and insulin levels (probably an attempt to compensate for an increased glycemic load) while, at a later stage, these mice more rapidly develop reduced β cell mass, lower insulin levels, and hyperglycemia. Thus, high mTORC1 activity in the pancreas is initially beneficial for glucose tolerance but leads to a faster decline in β cell function over time (Mori et al., 2009).

The role of mTORC1 in promoting muscle growth is well known but the mechanisms involved in this effect are still not clear. In mice muscles, mechanical stimuli, through Raptor phosphorylation IGF-1 and leucine, can activate mTORC1 and induce hypertrophy (Anthony et al., 2000; Rommel et al., 2001; Frey et al., 2014). Moreover, mice KO for mTOR and Raptor in muscle showed muscular atrophy and reduced body weight (Bodine et al., 2001; Rommel et al., 2001), suggesting a crucial role of mTORC1 in skeletal muscles. However, chronic mTORC1 hyperactivation in muscles results in severe muscle atrophy, low body mass, and early death, primarily due to an inability to induce autophagy in this tissue, where a normal turnover is fundamental to promote muscle growth.

Furthermore, many studies reveal a role for mTOR in promoting adipocyte formation and lipid synthesis in response to feeding and insulin. Nevertheless, mTORC1 inhibition in this tissue can have opposite effects, since adipocyte-specific Raptor KO mice showed lipodystrophy and hepatic steatosis (Lee et al.,
2016), but these mice are also resistant to diet-induced obesity due to reduced adipogenesis (Polak et al., 2008).

In addition, the study of rapamycin properties has elucidated its immunosuppressive activity, due to the ability of this drug to block T cell proliferation. In fact, different signals present in the immune microenvironment, such as interleukin-2 (IL-2) or amino acids too, can activate mTORC1, thus promoting T cells activation and expansion (Powell et al., 2012). MTORC1 inhibitors are currently used in immunosuppressive therapies but the mechanisms of action are very complex and still need to be addressed completely.

Finally, the phenotype of the hamartomatous syndrome tuberous sclerosis complex (TSC) patients has highlighted the role of mTORC1 hyperactivation in the brain. TSC is a genetic disease, caused by mutations of TSC1 or 2; patients exhibit a range of debilitating neurological disorders, including epilepsy, autism, and the presence of benign brain tumors. The strong correlation between mTORC1 hyperactivation and high occurrence of epileptic seizures and autism traits in TSC patients suggest a more general involvement of mTORC1 in these phenotypes. The importance of mTORC1 in this tissue is associated in part to its role in promoting activity-dependent mRNA translation near synapses, a critical step in neuronal circuit formation (Lipton and Sahin, 2014). Furthermore, autophagy dysfunction is strongly implicated in the pathogenesis of neurodegenerative disorders, including Parkinson’s disease and Alzheimer’s disease (AD). Accordingly, inhibition of mTORC1 signaling has beneficial effects on mouse models of AD (Spilman et al., 2010).
2.5 mTORC1 and cancer

As discussed above, mTORC1 represents the main node of a complex signaling network that controls cells, tissues and organismal growth. Since the activation of mTORC1 induces a cascade of anabolic processes associated to an high demand of nutrients and energy, this process is tightly regulated (Ilagan and Manning, 2016).

The first peculiar trait of cancer cells is the dysregulated growth caused by the loss of response to external stimuli, which, in physiological conditions, drive the activation/inactivation of mTORC1 (Ilagan and Manning, 2016). TSC1 and 2, known tumor suppressors and negative regulators of mTORC1 activity, represented the first link between mTORC1 hyperactivation and cancer (Crino et al., 2006). Moreover, mTORC1 is the downstream effector of several mutated oncogenic pathways, such as the PI3K/Akt pathway and the Ras/Raf/Mek/Erk (MAPK) pathway. Akt hyperactivation is a common oncogenic phenomenon that can result from PTEN deletion, PIK3CA activating mutations, the BCR-ABL translocation, and amplification of genes encoding HER-2, EGFR, or AKT itself (Guertin and Sabatini, 2005). For instance, Akt phosphorylates PRAS40, reducing its ability to inhibit mTORC1 (Sancak et al., 2007), but aberrant Akt activation can also result in the increase of TSC2 phosphorylation, which in turn relieves mTORC1 inhibition (Inoki et al., 2002). Other members of mTORC1 machinery have also been implicated in cancer progression: inactivating mutations in all the three subunits of GATOR have been found in glioblastomas (Bar-Peled et al., 2013); mutations in RagC were recently found, with an high frequency, in follicular lymphomas, causing its increased binding to Raptor, thus rendering mTORC1 signaling insensitive to amino acids deprivation (Okosun et al., 2016). Furthermore, FLCN mutations are the causative lesions of the Birt-Hogg-Dube
hereditary cancer syndrome, with a phenotype similar to TSC features (Nickerson et al., 2002), and MTOR mutations have been found in different cancer types (Grabiner et al., 2014).

The first mTORC1 inhibitors used in cancer therapy were a class of compounds derived from rapamycin and called "Rapalogs". The rapalog temsirolimus (Pfizer) was first approved for treatment of advanced renal cell carcinoma in 2007, followed by everolimus (Novartis) in 2009. These compounds were really promising in pre-clinical studies but the efficacy was not the same in humans, since only few groups of patients responded to the treatment. Different explanations were suggested to justify this failure. Rapalogs are allosteric inhibitors of mTORC1, thus they block the phosphorylation of some substrates, not all of them, and the phosphorylation of 4EBP1 is highly insensitive to rapamycin (Choo et al., 2008). In addition, increased Akt signaling has been observed in biopsies of cancer patients treated with everolimus because inhibiting mTORC1 releases the negative feedback on insulin/PI3K/Akt signaling and therefore may paradoxically promote cell survival and prevent apoptosis in some contexts (Tabernero et al., 2008). Moreover, mTORC1 inhibition induces autophagy and macropinocytosis, which can be a source of nutrients for tumors, promoting cancer cell survival (Rabinowitz and White, 2010; Perera et al., 2015), thus a combined treatment of mTORC1 inhibitors with autophagy inhibitors could have good outcomes. Subsequently, a second generation of Rapalogs has been developed: they directly block the catalytic activity of mTOR, suppressing both mTORC1 and mTORC2, but the prolonged treatment was still causing Akt reactivation, thus resulting no more effective (Rodrik-Outmezguine et al., 2011). Actually, for both first and second generation of mTOR inhibitors, the loss of efficacy is often associated to mutations in FKBP12–rapamycin-binding domain (FRB domain), causing reduced binding of
the first generation compounds, or mutations in mTOR kinase domain, resulting in inefficacy of the second generation inhibitors. Thus a third generation of mTOR inhibitors, called "RapaLinks", was developed in order to overcome the drug-resistance. These compounds are bivalent mTOR inhibitors consisting of a rapamycin–FRB-binding element appropriately linked to second-generation mTOR inhibitors. The exploitation of both the ATP- and the FRB-binding sites of mTOR allow the binding of the compound to one of the two binding domains, carrying the other half of the ligand in proximity to the second site, overcoming the reduced drug binding or the hyperactivity of the kinase, due to mutations in these domains (Rodrik-Outmezguine et al., 2016).
MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources: antibodies to human TFEB, phospho-T389 S6K1, S6K1, phospho-(Ser240/244) S6, S6, phospho-Ser757 ULK1, ULK1, phospho-(Ser 65) 4E-BP1, 4E-BP1, mTOR, RagA, RagB, RagC, RagD, FLCN, RAPTOR, ATG5, ATG7 and Histone H3 from Cell Signaling Technology; antibody to LAMP2 from Abcam; antibodies to TFE3, Actin and FLAG-BioM2 from Sigma Aldrich; antibody to murine TFEB from Bethyl laboratories; antibody to HA from Covance, antibody to LC3 from Novus Biologicals; antibody to P62 from Abnova; antibody to VAP-A was gently donated from De Matteis’ laboratory. MEM, DMEM, McCoy’s, RPMI, Fetal Bovine Serum (FBS) and Donkey Serum (DS) were from Euroclone; dialyzed FBS, Alexa 488 and 568-conjugated secondary antibodies, lipofectamine LTX and lipofectamine RNAimax were from Invitrogen; siRNAs were purchased as SMART pool from Dharmaco; torin 1, amino acids, MNase, polybrene were from Sigma Aldrich; puromycin was from Calbiochem; FuGENE 6, Complete Protease Cocktail and phosphatase inhibitors (Phospho Stop tablets) were from Roche; NeutrAvidin Agarose resin, SDS-OUT and D-biotin were from Pierce; amino acid-free RPMI from DBA; Mini-PROTEAN TGX Gels were from Biorad.

Cell culture and transfection

HeLa, HEK293T, HEPG2, U2OS, HK-2 cells were purchased from ATCC. Atg5-/-, Atg7-/- and WT MEFs cells were gently donated from Dr Maurizio Molinari. The
501Mel cells were a kind gift from Ruth Halaban (Halaban et al., 2000), and the A375P were from Colin Goding (Primot et al., 2010). HCR-59 cells were gently provided by Dr. Malouf (Malouf et al., 2014). Cells were cultured in the following media: HeLa in MEM; HEK293T and MEFs cells in DMEM high glucose; A375P, 501Mel and HEPG2 in RPMI; U2OS in McCoy’s; HK-2 in DMEM-F12 supplemented with 1% ITS; HCR-59 in RPMI supplemented with MEM non-essential amino acids (1X), 1% ITS, Hydrocortisone 0,04ng/ml and EGF 0,01 ug/ml. All media were supplemented with 10% FBS. Primary kidney cells were obtained following the protocol described in Calcagni et al. (Calcagni et al., 2016) and cultured in DMEM-F12 culture medium supplemented with 10% FCS, 1% ITS and 1% S1 hormone mixture. Plasmids were transfected with lipofectamine LTX and siRNA with lipofectamine RNAimax using a reverse transfection protocols and plated in 12 or 6-well dishes. After 48h of transfection with plasmids, cells were collected for RNA or protein analysis. Cells transfected with siRNAs (20nM) were collected after 72h since transfection.

**Western blotting**

Cells were lysed with ice-cold lysis buffer (TrisHcl 10mM pH 8.0-SDS 0.2% supplemented with protease and phosphatase inhibitors). Total lysate was briefly sonicated. Liver tissues were solubilized in homogenization buffer (25mM Tris-HCl pH 7.4, 10mM EDTA, 10mM EGTA, 1% NP40 supplemented with protease and phosphatase inhibitors). The soluble fractions from tissue lysates were isolated by centrifugation at 13,000 rpm for 10 minutes in a microfuge. From 10 to 30 micrograms of proteins were loaded on 10% or 4-15% Mini-PROTEAN TGX Gels transferred to PVDF membranes and analyzed by western blot using the ECL method (Pierce). Protein levels were quantified by using ImageJ software analysis.
Amino acid starvation/stimulation

Cells were rinsed with PBS and incubated in amino acid-free RPMI supplemented with 10% dialyzed FBS for 50 min, and then left untreated (0) or stimulated with increasing dosage of a mix of essential and non-essential amino acids for 15 min.

Organelle/cytosol fractionation

TFEB-CA HeLa cells were plated on 15cm plates and left untreated or treated with doxycycline. After 48h, they were rinsed with PBS and incubated in amino acid-free RPMI supplemented with 10% dialyzed FBS for 50 min, and then stimulated with a 3X amino acid mixture for 10min. Then, cells were rinsed with PBS and lysed using 10ml/dish of fractionation buffer (FB) (140mM KCl, 250mM Sucrose, 1mM DTT, 2mM EGTA, 2.5mM MgCl2, 25mM HEPES, pH 7.4) supplemented with 5mM glucose, protease inhibitor and 2.5mM ATP. After a brief centrifugation at 1700rpm for 10 minutes, pellet was resuspended in 750ul of FB and lysed by 23G needle; subsequently 750ul more of FB was added to the lysed and then it was centrifuged 10 min at 2700rpm in the 4C centrifuge. Post-nuclear supernatant (PNS) was transferred to a clean tube and centrifuged at 100K x g for 20min. The supernatant was the organelle-free cytosolic fraction. The pellet (organelles) was washed twice in buffer and eluted in SDS sample buffer. Equal fractions of pellets and supernatants were run on 4-15% Mini-PROTEAN TGX Gels for direct comparison.

Molecular biology

TFEB-WT and TFEB-CA TET-ON lentiviral plasmids were from Dr. Nick Platt; they carry the cDNAs for TFEB-WT or TFEB-S142A, S211A (TFEB-CA) 3X-FLAG
tagged and cloned into KpnI and NotI sites of pLVX-Tight-Puro vector (Clontech). 

**EGFP-TFE3** plasmid was acquired from Addgene and used as template to amplify TFE3 cDNA; this was subcloned into KpnI and NotI sites of pLVX-Tight-Puro vector. The cDNA for TFE3 was mutagenized to convert the two serins 246 and 321 in alanines using the QuickChange XLII mutagenesis kit (Stratagene).

Lentiviral plasmids for TFEB and TFE3 were co-transfected with Delta VPR envelope and CMV VSV-G packaging plasmids into actively growing HEK-293T cells using FuGENE 6 transfection reagent. Virus-containing supernatants were collected 48h after transfection, diluted 1:2 and used to infect a 100mm dish (50% confluent) of HeLa Tet-ON cells (Clontech) in presence of polybrene (4ug/ml). 24 hours later, cells were selected with puromycin and analyzed 7 days after infection.

Stable HeLa TFEB-CA and TFE3-CA were treated, where indicated, with doxycycline (1ug/ml) for 48 hours. Retroviral plasmids encoding TFEB-3X-FLAG or GFP (as control) were previously described (Settembre et al., 2012). They were co-transfected with pCMV-gag/pol and CMV VSV-G packaging plasmids into actively growing HEK-293T cells using FuGENE 6 transfection reagent. Virus-containing supernatants were collected 48h after transfection, diluted 1:2 and used to infect one six-well plate of MEFs in presence of 8ug/ml polybrene; then the plate was centrifuged at 2300 rpm RT for 90 min (spin infection). Cells were analyzed 3 days after infection.

The plasmid encoding human TFEB was previously described (Sardiello et al., 2009); the plasmids for HAGST-RagD and LAMP1-GFP were purchased from Addgene.
Luciferase assay

The promoter region for *RagD* spanning from CLEAR 3 to CLEAR5 was amplified by PCR from HeLa genome and subcloned into pGL3-basic luciferase reporter plasmid. Consensus sequences for the three CLEARs were mutagenized by using the QuickChange XLII mutagenesis kit. Plasmids were co-transfected together with increasing amount of *TFEB* plasmid and luciferase assays were performed 48 h after transfection using Dual Luciferase Reporter Assay System (Promega) and normalized for transfection efficiency by cotransfected Renilla luciferase.

Chromatin Immunoprecipitation assay (ChIP)

HeLa TET-ON TFEB-CA cells were treated with doxycycline for 48h or left untreated (control) and then crosslinked in 1% formaldehyde for 10 min and lysed on ice for 20 min in ChIP-Lysis buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1% Triton X-100, 1% Tween-20). After a 13 min MNase digestion (2U, Sigma-Aldrich) at 37°C, the reaction was stopped by addition of sodium dodecyl sulfate (SDS) and ethylenediaminetetraacetic acid (EDTA) to a final concentration of 1% and 2 mM, respectively. The unbound SDS of the cleared lysate was precipitated using SDS-OUT (Pierce, Rockford, IL, USA) to avoid compromising the immunoprecipitation. The lysates were diluted 1:1 with ChIPdilution buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.5% Triton X-100, 2 mM EDTA; all from Sigma-Aldrich) and preincubated with high capacity NeutrAvidin Agarose (Pierce). Protein-DNA complexes were immunoprecipitated for 4 h at 4°C with biotinylated FLAG antibody coupled to Neutravidin beads (2 mg ANTI-FLAG BioM2 from Sigma-Aldrich antibody with 50 ml Agarose slurry in ChIP-dilution buffer supplemented with 10 mg/ml BSA per sample). After three washings, the DNA
was eluted by addition of 8 mM biotin, 1% SDS in TE buffer. The DNA was precipitated after crosslink reversal using 200 mM NaCl at 65°C, overnight. One µl of DNA was used for each quantitative rtPCR reaction.

**RNA extraction, reverse transcription and quantitative PCR**

RNA samples from cells were obtained using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. RNA samples from mouse livers were extracted using TRlzl (Invitrogen) and re-purified with a RNeasy columns. cDNA was synthesized using QuantiTect Reverse Transcription kit (Qiagen).

Real-time quantitative RT-PCR on cDNAs was carried out with the LightCycler 480 SYBR Green I mix (Roche) using the Light Cycler 480 II detection system (Roche) with the following conditions: 95°C, 5 min; (95°C, 10 s; 60°C, 10 s; 72°C, 15 s) x 40. Fold change values were calculated using the DDCt method. Briefly, internal controls (HPRT1 or B2M for cell samples and Cyclophilin or S16 for mouse samples) were used as ‘normalizer’ genes to calculate the DCt value. Next, the DDCt value was calculated between the ‘control’ group and the ‘experimental’ group. Lastly, the fold change was calculated using 2(-DDCt). Biological replicates were grouped in the calculation of the fold change values.

**Immunofluorescence assays**

For detection of TFEB or TFE3, HeLa stable clones were grown on Lab-Tek chamber slides and treated with doxycycline for 48h. Cells were rinsed with PBS once and then: fixed for 15 min with 4% paraformaldehyde in PBS at RT, rinsed twice with PBS and then permeabilized with 0.2% Triton-X100 in PBS for 30min, and then incubated with blocking buffer (0.1% Triton-X100 plus 10% donkey
serum in PBS) for 1h. Subsequently, the slides were incubated with TFEB antibody (1:200) or TFE3 (1:500) in 0.1% Triton-X100 plus 5% donkey serum/PBS for 2h at RT, then rinsed three times with PBS and incubated with Alexa-Fluor conjugated secondary antibodies (Invitrogen) produced in donkey (diluted 1:700 in 0.1% Triton-X100 plus 5% donkey serum/PBS) for 1h at room temperature in the dark, washed four times with PBS. Slides were mounted on glass coverslips using Vectashield (Vector Laboratories) and imaged on a Leica SPE confocal microscope.

For detection of mTOR lysosomal localization: HeLa TFEB-CA cells were left untreated or treated with doxycycline for 48h; HeLa cells were transfected with scramble si-RNA or with siRNA for TFEB and then re-transfected with control plasmid and LAMP1-GFP plasmid, or with HAGSTragD and LAMP1-GFP plasmid. Cells were grown on Lab-Tek chamber slides and the day of experiment they were rinsed with PBS and incubated in amino acid-free RPMI supplemented with 10% dialyzed FBS for 50 min, and then left untreated or stimulated with a 3X amino acid mixture for 10min. Then, slides were rinsed with PBS once and fixed for 15 min with 4% paraformaldehyde in PBS at RT. After fixation, slides were rinsed twice with PBS and cells were permeabilized with Saponine 0.1% in PBS for 10min. After rinsing twice with PBS, the slides were incubated with primary antibody (mTOR and LAMP2, both 1:200) in 5% normal donkey serum for 1 hr at room temperature, rinsed four times with PBS, and incubated with secondary antibodies produced in donkey (diluted 1:700 in 5% normal donkey serum) for 45 min at room temperature in the dark, washed four times with PBS. Slides were mounted on glass coverslips using Vectashield (Vector Laboratories) and imaged on LSM 800 confocal microscope. Co-localization analysis was performed using ZEN 2008 software and it is expressed as colocalization coefficient. For detection
of LC3 on the lysosome, cells were fixed with 100% methanol and then processed as described above. Analysis was performed using ZEN 2008 software and it is expressed as LC3 Mean Intensity versus LAMP2 Mean Intensity in the co-localization region.

**Cell proliferation**

For MTT assay, 5mg of MTT powder was solubilized in 1mL of PBS and filtered. 10µL of this solution was added to 100µl of cell culture medium without phenol red. At the end of the incubation time, cells were washed twice with PBS and incubated with MTT-media solution to form formazan crystals. After two-three hours, media was removed and 100µl/well of a solubilization solution was added to the cells (2 mL H₂O AMMONIA in 50 mL DMSO) for 10 minutes at 37°C to obtain a complete solubilization of the crystals. As readout, absorbance of the 96-well plate was measured recording the Optical Density (OD) at 540nm with a microplate spectrophotometer system.

**Generation of RagD-promoter mutant HeLa cell line**

HeLa (ATCC CCL-2) cells carrying a homozygous deletion of the CLEAR-binding sequence in the promoter of the RagD gene were generated by using the CRISPR/Cas9 system. We selected the DNA region with the protospacer adjacent motif (PAM) sites within the CLEAR-box sequence GACCACGTGAA (-284) of the RagD promoter. The gRNA sequence (CTGTGCGGGGACCACGTGA) with low off-target score has been selected using the http://crispr.mit.edu/ tool. An “ALL in One” vector expressing Cas9, the specific gRNA and GFP was obtained from SIGMA (CAS9GFPP). The CAS9GFPP was nucleofected in HeLa cells using the
Amaxa (Cat No VCA-1003) and transfected GFP-positive cells were FACS sorted into 96 well plates to obtain single-cell derived colonies carrying the INDEL mutations. Upon genomic DNA extraction and DNA Sanger sequencing, a cell clone carrying a 33bps deletion (-266_299del TGCGGGACCACGTGAAGGAGAGGCGCGTGGGG) was selected and expanded.

**Mouse models**

All mice used were males and maintained in a C57BL/6 strain background. Mouse lines for conditional *Tcfeb-flox* was previously described (Settembre et al., 2012); *Albumin (Alb)-Cre* mice were obtained from the Jackson laboratory; *Myosin light chain 1 (Mlc)-Cre* mice were previously described (Bothe et al., 2000). The mouse line overexpressing Tcfeb in the kidney only was recently reported (Calcagnì et al., 2016). The *HDAd-TFEB* virus was described previously (Settembre et al., 2013a). Hepatic transduction was achieved by intravenous administration (retro-orbital) of 2x10\(^{13}\) viral particles/kg. Mice were analyzed one month after infection. The *RagD-AAV* vector was produced by the TIGEM AAV Vector Core Facility. Briefly, the human *HA-GST-RagD* coding sequence was cloned into the pAAV2.1-CMV-GFP plasmid by replacing the *GFP* sequence. The resulting pAAV2.1-CMV-HA-GST-RagD was then triple transfected in sub-confluent 293 cells along with the pAd-Helper and the pack 2/9 packaging plasmids. The recombinant AAV2/9 vectors were purified by two rounds of CsCl. Vector titers, expressed as genome copies (GC/mL), were assessed by both PCR quantification using TaqMan (Perkin-Elmer, Life and Analytical Sciences, Waltham, MA) and by dot blot analysis. As control virus, *GFP-AAV* vectors were used. Each mouse was retro-orbital injected with 1.25x10\(^{11}\) viral particles and sacrificed after 3 weeks. Experiments of
synchronization/fasting of mice were performed as previously reported (Ezaki et al., 2011); 30 minutes before to be sacrificed, mice were injected intraperitoneally (IP) with puromycin (21.8 mg/kg of mouse in 20 mM HEPES buffer pH 7.4). For exercise experiment, mice were let run for 1h at 25 cm/sec on a treadmill, for one week. On the last day of training, mice received oral gavage administration of leucine (1.35 g/kg of mouse in H2O); 30 minutes later they were injected IP with puromycin and after 30 minutes sacrificed for the analysis. Experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Cardarelli Hospital in Naples and authorized by the Italian Ministry of Health.

**Histology**

Livers were dissected, post-fixed with buffered 4% paraformaldehyde overnight at 4°C, then they were dehydrated in a graded series of ethanol, cleared with xylene, and infiltrated with paraffin. Paraffin-embedded blocks were cut on a microtome in 6-μm sections. Immunohistochemistry was performed using the Vectastain ABC kit (Vector Labs) following the manufacturer’s instructions. Signal was developed using 0.05% 3,3-diaminobenzidine tetrahydrochloride in 0.02% H2O2.

**Xenograft experiments**

Lentiviral particles (shLuc or shRagD) obtained from 293T transfection were added to 501Mel cells together with 4 μg/mL polybrene (Sigma) for 16 hours. After 48 hours, medium was replaced and 2μg/mL of puromycin was added for 72 hours before performing the experiment. 500,000 cells were resuspended in a 3:1 mix of cell medium and Matrigel Matrix (Corning 354248) and injected into the
subcutaneous abdominal space of NOD.Cg-Prkdcsid l12rgtm1Wjl/SzJ mice (NSG) mice. Tumor formation was monitored weekly. Mice were sacrificed when tumors of the control group (shLuc) reached the volume of \( \sim 0.5 \) cm\(^3\). In vivo data are presented as mean ± s.d. (standard deviation) from three independent experiments. NSG mice were purchased from Charles River. Mice of both sexes, 6-12 weeks old, were used for experimental procedures. In vivo studies were performed according to fully authorized animal facility, notification of the experiments to the Ministry of Health (as required by the Italian Law)(IACUCs No 758/2015) and in accordance to EU directive 2010/63.

**Statistics**

Student t test was used when comparing two groups; Anova was used when comparing more than two groups.
RESULTS

1. TFEB regulates mTORC1 activity.

In presence of nutrients, mTORC1 negatively regulates the activity of the MiT/TFE transcription factors by phosphorylating critical serine residues, thus causing their cytoplasmic retention (Martina et al., 2012; Roczniak-Ferguson et al., 2012; Settembre et al., 2011, 2012). Data from literature suggest that mTOR complexes may operate in the context of negative feedback loops to maintain various aspect of cellular homeostasis (Eltschinger et al., 2016), thus we postulated the presence of a feedback loop by which MiT/TFE transcription factors, which are substrates of mTORC1, may in turn influence mTORC1 activity.

To test whether TFEB could influence mTORC1 activity, we analyzed mTORC1 pathway upon amino acids stimulation in a HeLa cell line overexpressing the wild-type form of TFEB (TFEB-WT) and the constitutive active form of TFEB (TFEB-CA) in a doxycycline inducible manner. These cell lines were obtained by infection of TET-inducible lentiviral vectors in HeLA cells stably expressing the reverse tetracycline-controlled TransActivator (rtTA). The Tetracycline-ON system (Tet-ON) is activated by doxycycline, which binds to the rtTA promoting the transcription of the DNA sequences cloned downstream the Tet Response Element (TRE) (Gossen et al., 1995). We generated two TFEB-inducible HeLa cell lines, one expressing the WT form of TFEB and the other expressing the constitutive active form of TFEB (TFEB-CA), obtained mutating the serine residues 142 and 211 on TFEB coding sequence into alanines, thus relieving TFEB of mTORC1 inhibition (Martina et al., 2012; Roczniak-Ferguson et al., 2012; Settembre et al., 2011, 2012) (Fig.3).
Fig. 3. TetON system.

Scheme of doxycycline-inducible TFEB-WT and TFEB-CA constructs.

Upon doxycycline induction, the TFEB-WT cell line overexpressed the wild-type form of TFEB, showing a cytosolic localization in fed conditions and a nuclear localization in starved cells (Fig.4). Whereas the TFEB-CA cell line expressed a constitutively nuclear TFEB, in both fed and starved conditions (Fig.4). The two cell lines showed an increase in TFEB mRNA level of around 2 fold compared to non treated cells (Fig.5).

Fig. 4. TFEB subcellular localization in doxycycline-inducible Hela cell lines.

Representative immunofluorescence of TFEB in stable doxycycline-inducible cell lines, overexpressing the wild type form of TFEB (TFEB-WT) and TFEB-Const vitively Active (TFEB-CA).
Cells were untreated or treated with doxycycline (1mg/mL) for 48hours, in basal and in starved conditions. Scale bars 10 µm.

![Graph showing TFEB expression levels](image)

**Fig. 5. TFEB expression levels in doxycycline-inducible Hela cell lines.**

TFEB mRNA levels in TFEB-WT and TFEB-CA cells untreated or treated with doxycycline (1mg/mL) for 48hours. Gene expression was normalized relative to *HPRT1*. Values are mean ± SEM (*p < 0.05 Student t test).

We performed our studies preferentially in TFEB-CA cells, since this cell line represents a useful tool to escape mTORC1 regulation of TFEB. We observed that overexpression of TFEB-CA strongly affected mTORC1 activity in response to amino acids stimulation by looking at the phosphorylation of the main target proteins of mTORC1: direct targets, such as S6K1 and 4EBP1, and also indirect targets, like the ribosomal protein S6 (S6), phosphorylated by S6K1 (Fig.6).
Fig. 6. Analysis of phosphorylation levels of mTORC1 substrates in TFEB-CA cell line.

TFEB-CA cells, treated or untreated with doxycycline (1 mg/mL) for 48 hours, were starved for amino acids (a.a.) for 50 min and then left untreated (0) or stimulated with increasing levels of amino acids (expressed as % of a.a concentration in RPMI medium). Cell lysates were analyzed for phosphorylation of S6K, S6 and 4E-BP1 proteins. The plots represent mean values of triplicate experiments expressed as ratio of phosphorylated S6K versus pan-S6K, phosphorylated S6 versus pan-S6 and phosphorylated 4E-BP1 versus 4E-BP1. Values are mean ± SEM (*p < 0.05, **p<0.01 Student t test).

Conversely, TFEB depletion in HeLa cells significantly decreased mTORC1 activity upon amino acids administration (Fig.7).
Fig. 7. Analysis of phosphorylation levels of mTORC1 substrates in TFEB silenced cells.

Immunoblot analysis of S6K, S6 and 4E-BP1 phosphorylation in HeLa cells transfected with scramble or TFEB siRNA. Cells were first starved for amino acids (a.a.) for 50 min and then left untreated (0) or stimulated with increasing levels of amino acids (expressed as % of a.a concentration in RPMI medium). The plots represent mean values of triplicate experiments expressed as ratio of phosphorylated S6K versus pan-S6K, phosphorylated S6 versus pan-S6 and phosphorylated 4E-BP1 versus 4E-BP1. Values are mean ± SEM (*p < 0.05, **p<0.01 Student t test).

To confirm that these evidencies were not strictly associated to the cell line analyzed, we evaluated the phosphorylation status of mTORC1 substrates, after amino acids stimulation, in different cell lines transfected with TFEB or with a control vector. We analyzed respectively HEK293-T cells (Fig.8), HEPG2 cells (Fig.9) and U2OS cells (Fig.10) and in all of them we observed a striking upregulation of mTORC1 signaling upon TFEB overexpression compared to control samples. These data indicate that TFEB regulate mTORC1 signaling in different cell lines.
Fig. 8. TFEB regulates mTORC1 activity in HEK293-T cells.

Immuno-blotting analysis of threonine 389-S6K phosphorylation in HEK293-T cells transfected with TFEB or with a control pCDNA plasmid vector. Cells were starved for amino acids (a.a.) for 50 min and then left untreated (0) or stimulated with increasing levels of a.a. for 20 min.

Fig. 9. TFEB regulates mTORC1 activity in HEPG2 cells.

Immuno-blotting analysis of threonine 389-S6K phosphorylation in HEPG2 cells transfected with TFEB or with a control pCDNA plasmid vector. Cells were starved for amino acids (a.a.) for 50 min and then left untreated (0) or stimulated with increasing levels of a.a. for 20 min.
Fig.10. TFEB regulates mTORC1 activity in U2OS cells.

Immuno-blotting analysis of threonine 389-S6K phosphorylation in U2OS cells transfected with TFEB or with a control pCDNA plasmid vector. Cells were starved for amino acids (a.a.) for 50 min and then left untreated (0) or stimulated with increasing levels of a.a. for 20 min.

Since cell growth is a well known readout of mTORC1 activation (Saxton and Sabatini, 2017), we analyzed cell size and proliferation upon TFEB overexpression, to assess if TFEB levels could modulate these parameters. In line with our hypothesis, TFEB overexpression caused an increase in both cell size (Fig.11) and cell proliferation rate (Fig.12), and these effects were lost upon treatment with an inhibitor of mTORC1, Torin 1, suggesting that these results were due to TFEB-dependent mTORC1 hyperactivation.

Fig.11. Analysis of cell size upon TFEB overexpression.

FACS analysis performed in HeLa cells transfected with TFEB compared to control. Torin 1 was added, where indicated, for 48h (100nM). Plot represents means of three independent experiments ± SEM; one-way ANOVA.
Fig.12. Analysis of cell proliferation rate upon TFEB overexpression. MTT assay performed in HeLa cells transfected with TFEB compared to control. Torin was added, where indicated, for 48h (100nM). Plot represents means of three independent experiments ± SEM; one-way ANOVA.

These data, altogether, clearly indicate that TFEB regulates mTORC1 signaling pathway.

2. TFEB controls mTORC1 activity through RagD-GTPase.

Autophagy contributes to the pool of amino acids in the lysosomal lumen via lysosome-mediated proteolysis. Since TFEB belongs to the MiT/TFE family of transcription factors and it is an important regulator of autophagy (Sardiello et al. 2009, Settembre et al. 2011), we evaluated whether these factors regulate mTORC1 activity by modulating autophagy. Thus, we overexpressed TFEB in MEFs lacking the essential autophagy genes Atg5 (Fig.13) or Atg7 (Fig.14), and then we analyzed mTORC1 activity. TFEB overexpression in these autophagy-deficient cells still resulted in an enhanced mTORC1 activity, similar to the one observed in wild type MEFs, indicating that TFEB regulation of mTORC1 signaling is autophagy-indipendent (Fig.13, Fig.14).
**Fig.13. Analysis of mTORC1 activity in MEF Knock-Out for Atg5.**

Mouse embryonic fibroblasts (MEFs) knock out for Atg5 were retrovirally infected with a TFEB or control pCDNA plasmids. Cells were starved for a.a. for 50 min and then stimulated with increasing amount of a.a. for 20 min. Protein lysates were analyzed for the levels of threonine 389 S6K phosphorylation and the for the indicated proteins.

**Fig.14. Analysis of mTORC1 activity in MEF Knock-Out for Atg7.**

Mouse embryonic fibroblasts (MEFs) knock out for Atg7 were retrovirally infected with a TFEB or control pCDNA plasmids. Cells were starved for a.a. for 50 min and then stimulated with increasing amount of a.a. for 20 min. Protein lysates were analyzed for the levels of threonine 389 S6K phosphorylation and the for the indicated proteins.
To identify the mechanism responsible for TFEB control of mTORC1 activity, we investigated whether TFEB was regulating mTORC1 activity through transcriptional regulation of one or more genes involved in this pathway. Starting from a selection of 50 genes involved in mTORC1 activation (Table 1), we performed a bioinformatic analysis to look for CLEAR elements in the promoters of these selected genes. From this analysis, we obtained 20 putative TFEB target genes (Table 2) and we validated them by real time PCR in HeLa cells depleted for TFEB (Fig.15). The transcript levels of RagD, one of the RagGTPases involved in mTORC1 recruitment on the lysosomes (Sancak et al. 2008), were the most significantly downregulated in cells silenced for TFEB (Fig.15). Conversely, RagD was strongly induced upon doxycycline treatment in TFEB-CA cells (Fig.16). Similar results were obtained by western blot, where we observed a strong increase of RagD protein levels (Fig.17). Also RagC and FLCN transcript and protein levels were increased upon TFEB overexpression, albeit at a lower extent compared to RagD (Fig.16, Fig.17 ).
<table>
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</tr>
<tr>
<td>RRAGA</td>
<td>Ras related GTP binding A</td>
</tr>
<tr>
<td>RRAGB</td>
<td>Ras related GTP binding B</td>
</tr>
<tr>
<td>RRAGC</td>
<td>Ras related GTP binding C</td>
</tr>
<tr>
<td>RRAGD</td>
<td>Ras related GTP binding D</td>
</tr>
<tr>
<td>SEC13</td>
<td>SEC13 homolog, nuclear pore and COPII coat complex component</td>
</tr>
<tr>
<td>SEH1L</td>
<td>SEH1 like nucleoporin</td>
</tr>
<tr>
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</tr>
<tr>
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<tr>
<td>SESN3</td>
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<tr>
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</tr>
<tr>
<td>SLC38A9</td>
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</tr>
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<td>TSC2</td>
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</tr>
<tr>
<td>WDR59</td>
<td>WD repeat domain 59</td>
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**Table 1.** List of mTORC1 related genes analyzed for the presence of CLEAR elements in their promoter regions
Table 2. Distribution of CLEAR elements in the promoters of the selected mTORC1 related genes.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Score</th>
<th>Sequence</th>
<th>Chrom</th>
<th>Start</th>
<th>End</th>
<th>Strand</th>
<th>TSS Position</th>
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<td>AKT1S1</td>
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<td>17296385</td>
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Fig. 15. Expression levels of mTORC1 related genes in TFEB depleted cells.

mRNA levels of mTORC1-related genes in HeLa cells depleted for TFEB. B2M mRNA levels were measured as control gene. mRNA levels were normalized using HPRT1 and expressed as relative to cells transfected with scramble siRNA. B2M mRNA levels were measured as control gene. Values are normalized relative to HPRT1 and expressed as fold change relative to untreated cells. Bar graphs represent mean ± SEM of 3 independent experiments (*p < 0.05, **p < 0.01, Student t test).

Fig. 16. Expression levels of mTORC1 related genes in TFEB-CA cells.

mRNA levels of mTORC1-related genes in TFEB-CA HeLa cells treated with doxycycline (1mg/mL) for 48h. B2M mRNA levels were measured as control gene. Values are normalized relative to HPRT1 and expressed as fold change relative to untreated cells. Bar graphs represent mean ± SEM of 3 independent experiments (*p < 0.05, **p < 0.01, Student t test).
Fig. 17. **Protein levels of some of the mTORC1-related genes in TFEB-CA cells.**

Immuno-blotting analysis of the indicated proteins in TFEB-CA HeLa cells treated with doxycycline (1mg/mL) for 48h compared to control. The plot represents average values of triplicates normalized to histone H3 (*p < 0.05, **p < 0.01 Student t test).

RagD was strongly induced upon TFEB overexpression, both at transcript and at protein level, in different cell lines (HEK293-T, HEPG2, U2OS)(Fig. 18, Fig. 19).
Fig.18. Expression analysis of RagD gene in HEK293-T, HEPG2 and U2OS cells transiently transfected with TFEB plasmids.

Bars refer to fold changes of mRNA levels in TFEB-transfected cells versus cells transfected with control pCDNA plasmid. B2M levels were measured as control gene. Bar graphs represent mean ± SEM of 3 independent experiments (*p < 0.05, **p <0.01 Student t test).

Fig.19. Analysis of RagD protein levels in HEK293-T, HEPG2 and U2OS cells transiently transfected with TFEB plasmids.

Immuno-blotting analysis of RagD protein in lysates from HEK293-T, HEPG2 and U2OS cells transfected with TFEB or TFE3 compared to samples transfected with a control vector.

These data, altogether, support the hypothesis of RagD as the putative direct target of TFEB responsible for upregulation of mTORC1 signaling.

To confirm whether RagD was a direct target of TFEB, we performed chromatin immunoprecipitation (ChIP) experiments in our TFEB-CA cells. After the immunoprecipitation of the protein-DNA complexes, we performed a real time PCR using oligos amplifying the three CLEAR sites present in RagD promoter and we observed that TFEB binds to all three of them (Fig.20).
Fig. 20. TFEB directly binds to RAGD promoter.

ChIP analysis of TFEB binding to RAGD promoter in doxycycline-treated HeLa TFEB-CA cells. Cells were treated with doxycycline (1mg/mL) for 48h. Squares represent CLEAR sites in RAGD promoter and numbers refer to their distance [in base pairs (bp)] from the transcriptional start site (TSS). Immunoprecipitated DNA was normalized to the input and plotted as relative enrichment over a mock control.

Next, we performed luciferase assays amplifying by PCR in HeLa genome RAGD promoter from CLEAR 3 to CLEAR 5 and cloning it into a luciferase reporter plasmid. As negative control, we generated a construct carrying point mutations in the CLEAR sites, by using the QuickChange XLII mutagenesis kit, in order to limit TFEB binding to RAGD promoter. We observed a progressive increase in the luciferase activity of WT RagD-luciferase reporter vector upon transfection of increasing amounts of TFEB, while luciferase activity relative to the mutated vector was strongly blunted (Fig. 21).
Fig. 21. Analysis of TFEB binding to RagD promoter.

Luciferase assay after transfection of increasing amounts of TFEB construct was performed in HeLa cells cotransfected with wild-type (RAGD-wt) or mutated (RAGD-mut) RagD-promoter luciferase reporter plasmids. Plots represent mean ± SEM of three independent experiments (*p < 0.05; **p < 0.01; ***p < 0.001 Student’s t test).

As an additional evidence of the role of RagD in TFEB modulation of mTORC1 activity, we used a clustered regularly interspaced short palindromic repeats–Cas9 (CRISPR-Cas9) approach to remove the most responsive CLEAR element in RagD proximal promoter region in HeLa cells (HeLa-RagDpromedit) (Fig. 22).

Fig. 22. A CRISPR-Cas9 approach to edit RAGD promoter.

Scheme of CRISPR-Cas9–mediated mutation in the endogenous RAGD promoter of HeLa cells. A region of 33 bp containing the CLEAR site at position –284 (in red) was ablated.
This cell line showed reduced levels of RagD, both at mRNA (Fig. 23) and protein levels (Fig. 24), whereas the levels of the other RagGTPases were not affected. We observed no significant changes also for the mRNA and protein levels of FLCN (Fig. 23, Fig. 24).

![Graph showing mRNA levels of various genes in HeLa and HeLa-RagDpromedit cells.](image)

**Fig. 23.** RagGTPases and Folliculin (FLCN) mRNA levels in HeLa-RagDpromedit cells. Transcript levels of Rags and FLCN genes were analyzed in the mutated HeLa cell line (HeLa-RagDpromedit) versus control HeLa and normalized relative to HPRT1 gene. Plots represent mean ± SEM of three independent experiments (**p < 0.01 Student’s t test).**

![Western blot analysis of RagGTPases and FLCN levels in HeLa-RagDpromedit cells.](image)

**Fig. 24.** Western blot analysis of RagGTPases and FLCN levels in HeLa-RagDpromedit cells. Immuno-blotting analysis of RagGTPases and mTORC1-related proteins in three different samples of HeLa-RagDpromedit cells compared to control HeLa.

These data suggest that TFEB plays an important role in the regulation of RagD gene.
Furthermore, to understand if the reduced binding of TFEB to RagD promoter could affect mTORC1 activity in HeLa-RagD<sup>promedit</sup> cells, we analyzed the phosphorylation levels of mTORC1 canonical substrates in this cell line. We observed a significant reduction in mTORC1 signaling in stimulated HeLa-RagD<sup>promedit</sup> cells compared to control HeLa cells (Fig.25).

**Fig.25. Analysis of mTORC1 signaling in HeLa-RagD<sup>promedit</sup> cells.**

Immuno-blot of mTORC1 signaling in HeLa-RagD<sup>promedit</sup> cells compared with control HeLa. Cells were starved for amino acids (a.a.) for 50 min and then left untreated (0) or stimulated with increasing levels of amino acids (expressed as % of a.a concentration in RPMI medium). Cell lysates were analyzed for phosphorylation of S6K, S6 and 4E-BP1 proteins. The plots represent mean values of triplicate experiments expressed as ratio of phosphorylated S6K versus pan-S6K, phosphorylated S6 versus pan-S6 and phosphorylated 4E-BP1 versus 4E-BP1. Values are mean ± SEM (*p < 0.05, **p<0.01 Student t test).

The impairment of mTORC1 activity was then rescued upon overexpression of a plasmid carrying the wild type form of RagD (Fig.26).
Fig. 26. Analysis of mTORC1 signaling in HeLa-RagD\textsuperscript{promedit} cells transfected with RagD plasmid or with a control vector.

Immuno-blots of mTORC1 signaling in HeLa-RagD\textsuperscript{promedit} cells transfected with RagD plasmid or with a control vector. Cells were starved for amino acids (a.a.) for 50 min and then left untreated (0) or stimulated with increasing levels of amino acids (expressed as % of a.a concentration in RPMI medium). Cell lysates were analyzed for phosphorylation of S6K, S6 and 4E-BP1 proteins.

As a redout of mTORC1 decreased activity, we also analyzed the autophagy process in HeLa-RagD\textsuperscript{promedit} cells. Since mTORC1 inhibits autophagy (Ganley \textit{et al.} 2009, Hosokawa \textit{et al.} 2009), we expected to have increased autophagy levels in this cell line, due to the reduction of mTORC1 activity. We measured the protein levels of LC3 and P62, the commonly used markers of autophagy (He and Klionsky 2009), in HeLa-RagD\textsuperscript{promedit} cells and we observed increased LC3-II protein levels and reduced P62 levels, suggesting an increase in the autophagy process (Fig.27).
Fig. 27. Analysis of autophagy markers in HeLa-RagD promedit cells.

Western blot analysis of the indicated proteins in HeLa-RagD promedit cells relative to control cells. Cells were starved for amino acids (a.a.) for 50 min and then left untreated (0) or stimulated with increasing levels of amino acids for 20 min.

Furthermore, we performed LC3 co-staining together with LAMP2, a lysosomal membrane protein, to look at autophagosomes. Immunofluorescence images showed an increase of LC3-LAMP2 colocalization in HeLa-RagD promedit cells in starved conditions compared to HeLa control cells, indicating the presence of more autophagosome in HeLa-RagD promedit cells (Fig. 28). This colocalization coefficient still remained higher than the one in the control cells and almost similar to the values observed in starved conditions in HeLa-RagD promedit cells, after refeeding with amino acids, supporting the inability of mTORC1 to shut down autophagy in these cells (Fig. 28).
Representative immunofluorescence images of LC3 (green) and LAMP2 (red) in HeLa-RagDpromedit and control HeLa cells starved or starved and then re-fed for 2 hour with nutrient rich media. The plot shows LC3-LAMP2 co-localization values. n= 20 cells/condition from three independent experiments (mean ± SEM, *p <0.05 Student t-test). Scale bars 20 µm.

Hence, these data altogether clearly indicate that TFEB transcriptionally regulate RagD gene, supporting a prominent role of this RagGTPase in TFEB regulation of mTORC1.

3. TFEB promotes mTORC1 recruitment to the lysosome.

RagGTPases are involved in mTORC1 recruitment on the lysosomal membrane, interacting with the subunit Raptor of the mTORC1 complex (Sancak et al., 2008; Kim et al., 2008). Thus, we decided to evaluate if TFEB levels could affect mTORC1 lysosomal localization.

Immunofluorescence analysis, performed in TFEB-CA cells, showed an increase colocalization coefficient of mTOR and the lysosomal marker LAMP2, upon amino acids stimulation, compared to control cells (Fig.29).
Fig. 29. mTOR localization in TFEB-CA cells.

Representative immunofluorescence images of mTOR and LAMP2 in TFEB-CA HeLa cells left untreated or treated with doxycycline (DOXY, 1mg/mL for 48h). Cells were amino acids deprived for 50 minutes and then stimulated with amino acids for 15 minutes. The plot represents quantification of the data from 15 cells per condition from three independent experiments. Results are shown as means of co-localization coefficient of mTOR and LAMP2 ± SEM (**p <0.01, Student t test). Scale bars 10 µm.

To support the immunofluorescence data obtained, we performed organelle/cytosol fractionation experiments in TFEB-CA overexpressing cells compared to control. This biochemical assay showed an enrichment of mTORC1 components in the organelle fraction after TFEB overexpression, indicating an increased presence of mTORC1 in the pool containing also the lysosomes (Fig.30).
**Fig. 30. Organelle/cytosolic fractionation in TFEB-CA cells.**

Western blot analysis of the indicated proteins in membranes and cytosolic fractions isolated from TFEB-CA HeLa cells left untreated or treated with doxycycline (1mg/mL) for 48h. LAMP2 and VAP-A were used as loading control for membrane fractions, tubulin for cytosolic fractions.

Conversely, we observed a reduction in mTORC1 recruitment on the lysosomes in cells depleted for TFEB after amino acids stimulation (Fig. 31). This phenotype was rescued by the overexpression of a RagD-HA plasmid in these cells: relocalization of mTOR on the lysosomal membrane was visible only in cells silenced for TFEB but positive for the HA tag (Fig. 31).
**Fig.31.** mTOR localization in cells depleted for TFEB, transfected with control vectors or with RagD-HA plasmid.

Representative immunofluorescence images of endogenous mTOR, LAMP1-GFP (visualized as red) and RAGD-HA (in purple) in HeLa cells. Cells were transfected with scramble (CTRL) or with TFEB siRNA (siTFEB) and after 48 hours with LAMP1-GFP and with RagD-HA plasmids for an additional 24 hours. Cells were deprived of amino acids for 50 min and then stimulated with amino acids for 15 min. Plots represent quantification of the data from 15 cells per condition from three independent experiments. Results are shown as means of colocalization coefficient of mTOR and LAMP2 ± SEM (**p < 0.01 Student’s t test). Scale bars, 10 mm.

Finally, we analyzed mTOR lysosomal localization in HeLa-RagDpromed cells and found a significant reduction in mTOR recruitment to the lysosome relative to control HeLa cells upon amino acid stimulation (Fig.32).

**Fig.32.** mTOR localization in HeLa-RagDpromed cells.

Representative immunofluorescence images of mTOR and LAMP2 in HeLa-RagDpromed and in control HeLa cells. Cells were deprived of amino acids for 50 min and then stimulated with amino acids for 15 min. Plots represent quantification of the data from 15 cells per condition from three independent experiments. Results are shown as means of colocalization coefficient of mTOR and LAMP2 ± SEM (***p< 0.001 Student’s t test). Scale bars, 10 mm.
These data clearly indicate that TFEB-mediated transcriptional regulation of RagD promotes an efficient recruitment of mTORC1 complex to the lysosome upon nutrient stimulation.

4. Nutrient-induced mTOR reactivation after starvation and exercise is mediated by TFEB

TFEB is a nutrient-sensing transcription factor: in starvation conditions, it translocates into the nucleus and activates its target genes involved in catabolic processes (Settembre et al., 2011). To understand if RagD could participate to the starvation response, we analyzed RagD expression levels in cells deprived for amino acids and we observed an increase in RagD mRNA levels, around 2 fold compared to control, that was blunted in cells depleted for TFEB (Fig.33).

![Fig.33. RagD expression levels in starvation.](image)

mRNA levels of RagD in HeLa cells transfected with scramble (CTRL), TFEB siRNAs and kept in basal medium or starved for amino acids (a.a.) for 4 hours. Bars represent RagD mRNA levels in the indicated cells/conditions expressed as fold changes relative to cells transfected with scramble siRNA kept in basal medium. The plot represents mean ± SEM of 3 independent experiments (**p < 0.01 Student t test).
Similarly, RagD protein levels were increased after 4 hours of amino acids starvation, and this increase was lost in cells silenced for TFEB (Fig. 34). There were no significant changes in the levels of the other RagGTPases and of FLCN (Fig. 34).

![Image](image.png)

**Fig. 34.** Analysis of mTORC1 activity and mTORC1-related protein levels in relation to nutrients availability and to TFEB levels.

Immunoblot analysis of S6K phosphorylation and of mTORC1-related proteins in HeLa cells transfected with CTRL (scramble) or TFEB siRNA, kept in basal medium (bas.), starved for 4 hours (starv.) or starved and then refed (refed) for 6 hours.

Furthermore, we followed TFEB localization in TFEB-WT and in TFEB-CA inducible cells in response to nutrients availability (Fig. 35) and *RagD* gene expression levels in the same conditions (Fig. 36). In TFEB-WT cells, TFEB localization responds to nutrient conditions: it resides into the cytoplasm when nutrients are available, while it translocates into the nucleus upon amino acid starvation. Instead, in TFEB-CA cells, TFEB is always localized into the nucleus (Fig. 35). Importantly, we found that RagD expression levels correlated with TFEB cellular localization (Fig. 36).
These data suggest that TFEB is driving RagD upregulation in starvation conditions.

Fig.35. TFEB subcellular localization in TFEB-WT and TFEB-CA cells.
Immunofluorescence analysis of TFEB localization in TFEB-CA, TFEB-WT and control (CTRL) HeLa cells kept in basal condition (basal), starved for amino acids for 2 and 4 hours (STV-2h, STV-4h), or starved for 4 hours and stimulated with complete medium for 6 (RE-FED-6h) and 12 hours (RE-FED-12h). Cells were treated with doxycycline (1mg/mL) for 48h. Scale bars 10 µm.

Fig.36. RagD expression levels in response to nutrient availability in TFEB-WT and TFEB-CA cells.
mRNA levels of RagD in TFEB-CA (grey lines), TFEB-WT (brown lines) and in control (black lines) HeLa cells in response to nutrient starvation/stimulation at the indicated times. The plot represents
mean ± SEM of 3 independent experiments. Values were normalized to HPRT1 and expressed as relative to basal conditions. (*p < 0.05, **p < 0.01, ***p < 0.001 Anova (two-way)). Scale bar is logarithmic. Cells were treated with doxycycline (1mg/mL) for 48h.

To unravel the physiological relevance of TFEB-mediated transcriptional regulation of RagD we move in vivo. We first analyzed RagD mRNA levels in the liver of wild type mice that were fasted for different timepoints. Similarly to what observed in cell cultures, fasting in mice induced upregulation of RagD expression in the liver (Fig.37).

Fig.37. Upregulation of RagD expression in the liver of WT mice fasted.

mRNA levels of RagD in C57BL6 WT mice fasted for the indicated times. Bar graph shows values (means ± SEM for n=3 mice) expressed as fold increase compared to fed mice (*=p < 0.05, **p < 0.01 Student t test).

Moreover, we injected C57BL6 WT mice with an Helper-Dependent-Adenovirus (HD-Ad) expressing human TFEB under the control of a liver-specific promoter (PEPCK) or with control virus. One month after injection, mice were fasted for 24 hours or nutritionally synchronized by fasting them for 22 hours and then giving the food back for 2 hours, and later on they were sacrificed. Liver samples from the different murine groups were analyzed by RT-PCR and we found a strong
increase in RagD transcript levels upon TFEB injection, which was even stronger in fasted mice (Fig.38).

Fig.38. RagD mRNA levels in TFEB-injected mice.
C57BL6 mice injected with a Helper-Dependent Adenovirus that expresses human TFEB (HDAd-TFEB) under the control of a liver-specific promoter (PEPCK), or with saline PBS (control) were starved for 22h, then refeed for 2h (FED), or starved for additional 12 h (FASTED) prior to sacrifice. Liver tissues were analyzed for mRNA levels of RagD. Bar graph shows values (means ± SEM for n=5 mice) expressed as fold increase compared to control mice (**=p < 0.01 Student t test).

Accordingly, western blot analysis of liver samples from the same groups of mice showed increased mTORC1 signaling activation in TFEB-Injected mice upon nutrient synchronization (Fig.39). This was confirmed by immunohistochemistry analysis of S6-phosphorylation in TFEB-injected versus control mouse tissues (Fig.40). Notably, signal for S6-phosphorylation correlated with the one for TFEB in transduced hepatocytes (Fig. 40 insets).
Fig. 39. Analysis of mTORC1 substrates in TFEB-Injected mice.
C57BL6 mice injected with HDAd expressing human TFEB under the control of a liver-specific promoter (TFEB-INJ) or with phosphate-buffered saline (PBS) (CTRL) were starved for 22 hours and then refed for 2 hours (FED), or starved for additional 12 hours (FASTED). Liver lysates were analyzed for levels of indicated proteins. Actin was used as loading control. Plot shows ratio of phosphorylated S6K/pan-S6K (mean of three independent experiments).

Fig. 40. Analysis of mTORC1 signaling by immunohistochemistry in TFEB-Injected mice.
Immunohistochemistry of liver sections from mice injected with saline PBS (CTRL) or HDAd-TFEB (TFEB-INJ). Tissues were stained for serine 240/244 phosphorylated-S6 (P-S6). Insets show overlapping P-S6 and TFEB immunostainings in two consecutive 5-mm liver sections isolated from HDad-TFEB-injected mice.
Next, we analyzed the consequences of TFEB depletion on mTORC1 signaling in vivo, taking advantage of murine models already available in the lab. Due to the embrional lethality of Tcfb KO mice (Steingrimsson et al., 1998), several tissue-specific conditional Tcfb KO models were generated. We chose to study Tcfb liver-specific conditional knockout (KO) mice (Tcfb<sup>fl<sup>ox</sup></sup>/fl<sup>ox</sup>; Alb-CRE<sup>+</sup>; hereafter Tcfb-LiKO) and Tcfb muscle-specific KO mice (Tcfb<sup>fl<sup>ox</sup></sup>/fl<sup>ox</sup>; Mlc-CRE<sup>+</sup>; hereafter Tcfb-MuKO), since mTORC1 has a prominent role in regulating liver and muscle metabolism. Tcfb<sup>-flo</sup> mice were described in Settembre et al. 2012 and muscle specific Cre mice (Mlc-Cre mice) were described in Bothe et al. 2000, whereas liver specific Cre mice (Alb-Cre mice) were obtained from the Jackson laboratory. Accordingly to what we observed previously, we could detect an increase in RagD expression levels upon fasting, but this effect was blunted in Tcfb-LiKO mice (Fig.41).

**Fig.41. RagD mRNA levels are reduced in Tcfb-LiKO fasted mice.**

Transcript levels of RagD in liver tissues isolated from mice with indicated genotypes fasted for 24 hours. Values were normalized to Cyclophilin gene and expressed as fold change relative to control fed mice. Bars represent means ± SEM (n=5 mice/group; **=p < 0.01 Anova (one-way) followed by Tukey’s test).
Furthermore, we observed a reduction in mTORC1 activity in Tcfeb-LiKO mice compared to controls (Tcfeb\textsuperscript{flox/flox} mice), as shown by the decrease in the phosphorylation of S6K(Thr389) in these mice relative to control mice (Fig.42). Additionally, we followed protein synthesis, a well known redout of mTORC1 activity. Mice were nutritionally synchronized and injected with puromycin 30 minutes prior to sacrifice. Puromycin gets incorporated in the nascent polypeptides, thus allowing to follow protein synthesis by western blot analysis using an antibody against puromycin. We observed a strong decrease in puromycin signal in samples from Tcfeb-LiKO mice, compared to control, indicating a reduced protein synthesis (Fig.42). Both the decreased phosphorylation of S6K(Thr389) and the impairment of protein synthesis, observed in Tcfeb-LiKO mice, were rescued by viral-mediated delivery of human RagD gene, by using an Adeno-Associated Virus serotype 2/9 (AAV-2/9) (Fig.42).

**Fig.42. mTORC1 activity is reduced in Tcfeb-LiKO mice.**

Mice with indicated genotypes were nutritionally synchronized and injected with puromycin 30 min before sacrifice. Where indicated, Tcfeb\textsuperscript{floxfloxflox}, Alb-Cre\textsuperscript{+} mice were injected with an adeno-associated virus vector carrying human RagD cDNA. Liver lysates were analyzed for...
Subsequently we analyzed the Tcfeb-MuKO mice. It is reported that a protein-rich-meal after exercise increases the rate of muscle protein synthesis via activation of mTORC1 signaling, even if the signaling pathways underlying this mechanism are not yet fully understood (Watson and Baar, 2014). Moreover, exercise is known to promote TFEB nuclear translocation in muscle via calcineurin-mediated dephosphorylation (Medina et al., 2015). Tcfeb-MuKO mice and control mice (Tcfeb^flox/flox) were subjected to physical exercise or kept in resting condition. For exercise experiments, mice were let run for 1h at 25cm/sec on a treadmill, for one week. On the last day of training, mice received oral gavage administration of leucine (1,35g/kg of mouse in H2O); 30 minutes later they were injected intraperitoneally (IP) with puromycin and after 30 minutes sacrificed. Analysis of muscles samples from the different murine groups revealed that physical exercise induced RagD expression in control mice, but this response was blunted in Tcfeb-MuKO mice (Fig.43).
Fig. 43. RagD upregulation induced by the exercise is reduced in Tcfeb-MuKO mice.
mRNA levels of RagD in exercised mice with indicated genotypes. Values were normalized to cyclophilin gene. Bars represent means ± SEM for n=4 mice and are expressed as fold change relative to control rested mice (**=p < 0.01; Anova (one-way) followed by Tukey’s test).

Moreover, we observed an impairment in mTORC1 activation and in the protein synthesis in response to the protein meal after exercise in Tcfeb-MuKO mice compared to controls, represented respectively in the reduction of the phosphorylation levels of S6K (Thr389) and in the decreased puromycin signal detected (Fig. 44).
Fig.44. mTORC1 activity, induced by physical exercise, is impaired in Tcfeb-MuKO mice.

Phosphorylation of S6K and levels of puromycin incorporation analysis in muscle samples from mice with indicated genotypes after oral gavage of leucine. Mice were exercised where indicated. Plots show ratios of phosphorylated S6K/pan-S6K and puromycin/glyceraldehyde phosphate dehydrogenase (GAPDH). Plots in represent means ± SEM; N = 3/condition; one-way analysis of variance (ANOVA) followed by Tukey’s test. *p < 0.05; **p < 0.01.

Together these results suggest that amino acid-induced mTORC1 signaling after starvation and exercise is strongly influenced by TFEB via the transcriptional regulation of RagD.

5. MiT/TFE family factors, and not only TFEB, regulate mTORC1 activity through RagD-GTPase.

MiT/TFE transcription factors family members, TFEB, TFE3 and MITF, recognize the same E-box sites in the proximal promoter regions and therefore they can regulate the same target genes (Steingrímsson et al., 2004; Sardiello et al., 2009).
To test whether, not only TFEB, but also TFE3 and MITF, could control mTORC1 activity, we repeated some of the experiments performed for the characterization of TFEB role in mTORC1 signaling, in the same conditions, but pointing the attention to the other two factors.

Regarding TFE3, as we did it for TFEB, we generated a doxycycline-inducible cell line overexpressing the wild-type form of TFE3 (TFE3-WT) and its constitutively active form (TFE3-CA), where we mutated the serine residues 246 and 321, phosphorylated by mTORC1 (Martina et al., 2014), into alanines. Upon doxycycline induction, the TFE3-WT cell line overexpressed the wild-type for of TFE3 with a subcellular localization in line with the nutrient availability, whereas the TFE3-CA cell line expressed a constitutively nuclear TFE3 (Fig.45). Both cell lines showed an increase in TFE3 mRNA levels of around 2 fold compared to untreated cells (Fig.46).

Fig.45. TFE3 subcellular localization in doxycycline-inducible Hela cell lines.
Representative immunofluorescence of TFE3 in stable doxycycline-inducible cell lines, overexpressing the wild type form of TFE3 (TFE3-WT) and TFE3- Constitutively Active (TFE3-CA). Cells were untreated or treated with doxycycline (1mg/mL) for 48 hours, in basal and in starved conditions. Scale bars 10 µm.
TFE3 mRNA levels in TFE3-WT and TFE3-CA cells untreated or treated with doxycycline (1mg/mL) for 48hours. Gene expression was normalized relative to HPRT1. Values are mean ± SEM (*p < 0.05 Student t test).

Subsequently, we analyzed mTORC1 signaling, upon amino acids administration, in TFE3-CA cells, and we observed an increased phosphorylation of all mTORC1 canonical substrates, as observed in TFEB-CA cells (Fig.47).
for phosphorylation of S6K, S6 and 4E-BP1 proteins. The plots represent mean values of triplicate experiments expressed as ratio of phosphorylated S6K versus pan-S6K, phosphorylated S6 versus pan-S6 and phosphorylated 4E-BP1 versus 4E-BP1. Values are mean ± SEM (*p < 0.05 Student t test).

Conversely, we detected a reduction in mTORC1 signaling, upon amino acids stimulation, in cells depleted for TFE3 (Fig.48), indicating that also TFE3 is involved in mTORC1 regulation.

![Fig.48. Analysis of phosphorylation levels of mTORC1 substrates in TFE3 silenced cells.](image)

Immunoblot analysis of S6K, S6 and 4E-BP1 phosphorylation in HeLa cells transfected with scramble or TFE3 siRNA. Cells were first starved for amino acids (a.a.) for 50 min and then left untreated (0) or stimulated with increasing levels of amino acids (expressed as % of a.a concentration in RPMI medium). The plots represent mean values of triplicate experiments expressed as ratio of phosphorylated S6K versus pan-S6K, phosphorylated S6 versus pan-S6 and phosphorylated 4E-BP1 versus 4E-BP1. Values are mean ± SEM (*p < 0.05 Student t test).
Moreover, RagD was the most significantly down-regulated gene in cells depleted for TFE3 (Fig.49), suggesting that, similarly to TFEB, TFE3 regulates mTORC1 activity through RagD.

![Fig.49. RagD mediates TFE3 regulation of mTORC1 activity.](image)

Expression analysis of mTORC1-related genes in HeLa cells depleted for TFE3. B2M mRNA levels were measured as control gene. mRNA levels were normalized using HPRT1 and expressed as relative to cells transfected with scramble siRNA. Bar graphs represent mean ± SEM of 3 independent experiments (*p < 0.05, **p < 0.01 Student t test).

Then, we transiently overexpressed MITF in HeLa cells and we evaluated mTORC1 activity, upon amino acids stimulation, (Fig.50), and RagD mRNA levels (Fig.51), and we found that both were strongly induced in MITF overexpressing cells relative to control cells (Fig.50, Fig.51).
**Fig. 50. mTORC1 activity is increased in MITF overexpressing cells.**

HeLa cells, transfected with a vector encoding MITF or with an empty vector, were starved for amino acids (a.a.) for 50 min and then left untreated (0) or stimulated with increasing levels of amino acids (expressed as % of a.a concentration in RPMI medium). Cell lysates were analyzed for phosphorylation of S6K, S6 and 4E-BP1 proteins.

**Fig. 51. RagD transcription is upregulated in MITF overexpressing cells.**

HeLa cells were transfected with a vector encoding MITF or with an empty vector and analyzed for RagD mRNA levels. Bar graphs represent fold change of mRNA levels in MITF-transfected cells relative to cells transfected with control vector. Values represent means ± SEM, N=3 (***p < 0.001 Student t test).

In conclusion, these results support our hypothesis that MiT/TFE factors share the ability to control mTORC1 via RagD transcriptional regulation.
6. Deregulation of the MiT/TFE-RagD-mTORC1 regulatory axis supports cancer growth.

MiT/TFE factors are known oncogenes, overexpressed in a variety of tumors such as renal cell carcinoma (RCC), melanoma, sarcoma, and pancreatic ductal adenocarcinoma (Haq and Fisher, 2011; Kaufmann et al., 2014; Perera et al., 2015). Furthermore, mTOR pathway is highly implicated in cancer pathogenesis (Laplante and Sabatini, 2012). Due to these evidences, we decided to characterize MiT/TFE-RagD-mTORC1 regulatory axis in tumors overexpressing the MiT/TFE factors.

It was already available in our lab a Tcfeb kidney-specific conditional overexpressing transgenic mouse model that displayed a phenotype similar to human RCC (Calcagnì et al., 2016). We thus analyzed mTORC1 signaling both in kidney tissues and primary kidney cells obtained from these mice, relative to control mice. We found hyperactivation of mTORC1 signaling in terms of increased signal of P-S6 staining, by immohistochemistry, in transgenic mice compared to controls (Fig.52), and primary kidney cells derived from transgenic mice displayed an increased phosphorylation of mTORC1 substrates upon amino acids stimulation (Fig.53).
Fig. 52. mTORC1 hyperactivity in kidney tissues from TFEB kidney-specific conditional overexpressor mice.

Representative images of kidney sections from controls (CTRL, Cond-tgTcfeb<sup>+</sup>−<sup>−</sup>) and TFEB kidney-specific conditional overexpressor mice (KSPcdh16<sup>+/−</sup>;Cond-tgTcfeb<sup>−<sup>−</sup>−</sup> indicated as TG) stained for serine 240/244 phosphorylated-S6 (P-S6) ribosomal protein at post-natal days 14 (P14) and P30.

Fig. 53. Increased phosphorylation of mTORC1 substrates in primary kidney cells from TFEB kidney-specific conditional overexpressor mice.

Representative immuno-blotting analysis of phosphorylation levels of the indicated mTORC1 substrates in control and in TFEB-overexpressing primary kidney cells. Actin was used as loading control.

We then evaluated RagD expression levels in primary kidney cells obtained from these mice. As expected, we found increased RagD transcript levels in TFEB-overexpressing primary kidney cells compared to control cells (Fig. 54).
Fig.54. RagD transcription is increased in TFEB-overexpressing kidney primary cells.

RagD mRNA levels in primary kidney cells isolated from TFEB kidney-specific conditional overexpressor mice expressed as fold change relative to control mice. Gene expression was normalized relative to Cyclophilin; S16 expression was shown as control unrelated gene. Bar graph shows mean ± SEM of 3 independent experiments (**p < 0.01 Student t test).

Furthermore, primary kindney cells from transgenic mice presented an hyperproliferative phenotype, revertable by Torin 1 treatment (Fig.55), indicating that this phenotype was associated to mTORC1 hyperactivation.

Fig.55. TFEB-overexpressing primary kidney cells hyperproliferation is mTORC1 dependent.

MTT assay was used to measure cell proliferation of TFEB-overexpressing primary kidney cells (TG) compared to control cells (CTRL). Torin 1 was added, where indicated, for 48h (100nM). The plot represents means of three independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001 Student t test).
Successively, we analyzed kidney cells derived from a RCC-patient carrying a chromosomal translocation that involves the TFE3 gene (HCR-59 cell line) (Malouf et al., 2014). HCR-59 cells showed increased RagD transcript (Fig. 56) and protein levels (Fig. 57) and enhanced mTORC1 signaling relative to HK2 kidney cell line (Fig. 57).

**Fig. 56. RagD transcription is increased in HCR-59 cells.**

mRNA levels of RagD in a cell line from a patient with RCC (HCR-59) relative to control kidney cells (HK-2). B2M expression shown as control unrelated gene. Gene expression was normalized relative to HPRT1. Plot represents means of three independent experiments ± SEM (**p< 0.01 Student's t test).
Fig.57. mTORC1 signaling and RagD protein levels are increased in HCR-59 cells.
Analysis of S6K phosphorylation at threonine 389 in HK-2 and HCR-59 cells 50 min starved for amino acids (0) and then stimulated with increasing levels of amino acids for 20 min.

Silencing of both TFE3 (Fig.58) or RagD (Fig.59) reduced the increased phosphorylation of mTORC1 substrates, upon amino acids administration in HCR-59 cells, indicating that both TFE3 and RagD are implicated in mTORC1 hyperactivity.

Fig.58. mTORC1 hyperactivation is reduced in HCR-59 depleted for TFE3.
HCR-59 cells were transfected with scramble (siCTRL) or TFE3 siRNA and then analyzed for the indicated proteins upon stimulation with increasing % of amino acids.

Fig.59. mTORC1 hyperactivation is reduced in HCR-59 depleted for RagD.
HCR-59 cells were transfected with scramble (siCTRL) or RagD siRNA and then analyzed for
phosphorylation levels of S6K upon stimulation with increasing % of amino acids.

Moreover, HCR-59 cells, silenced for TFE3 or RagD, showed also reduced proliferation rate (Fig.60), indicating that the MiT/TFE-RagD-mTORC1 axis deregulation is responsible for the tumor cell proliferation in RCC.

![Fig.60](image)

**Fig.60. Hyperproliferation of HCR-59 cells is reduce upon depletion of TFE3 or RagD.**

Proliferation levels of HCR-59 cells transfected with scramble (SCR), RagD, or TFE3 siRNAs. Plot represents means of three independent experiments ± SEM; one-way ANOVA. (***p < 0.001, Student's t test).

MITF, the MiT/TFE family member involved in melanosomal biogenesis (Hodgkinson *et al.*, 1993), has an oncogenic role well established in melanoma (Tsao *et al.*, 2012). To understand if the MiT/TFE-RagD-mTORC1 axis deregulation could be involved in melanoma pathogenesis, we analyzed a melanoma cell line derived from a patient (501Mel) presenting MITF overexpression. 501Mel cells showed increased RagD mRNA (Fig.61) and protein levels (Fig.62) and upregulation of mTORC1 signaling upon amino acids stimulation, compared to control melanoma cells non-overexpressing MITF (A375P) (Fig.62). Thus, also in MITF-related-melanomas we observed a strong
increase of mTORC1 signaling pathways, correlating with a strong upregulation of RagD transcript levels.

![Graph showing fold change in RagD transcript levels](image)

**Fig.61. RagD transcription is increased in 501Mel cells.**

MITF-dependent melanoma patient–derived cells (501Mel) were analyzed for mRNA levels of *RagD* (*B2M* expression was shown as control unrelated gene). Values expressed as relative to control melanoma cells (A375P). Gene expression was normalized relative to *HPRT1*. Plot represents means of three independent experiments ± SEM (**p < 0.001, Student's t test).

![Immunoblotting analysis](image)

**Fig.62. mTORC1 signaling and RagD protein levels are increased in 501Mel cells.**

Representative immunoblotting analysis for the indicated proteins in control (A375P) and MITF-dependent melanoma (501Mel) cells stimulated with increased levels of amino acids.

Next, we depleted MITF in 501Mel cells and analyzed mTORC1 signaling after amino acids stimulation. We observed a decrease in the phosphorylation of S6K (Thr389) and also a reduction of RagD protein levels (Fig.63), supporting the
prominent role of the MITF-RagD-mTORC1 axis deregulation in this melanoma cells.

Fig. 63. mTORC1 hyperactivation is reduced in 501Mel depleted for MITF.

501Mel cells were transfected with scramble (siCTRL) or MITF siRNA and then analyzed for the protein indicated upon stimulation with increasing % of amino acids.

Furthermore, the down-regulation of MITF or RagD rescued the hyperproliferative phenotype displayed by 501Mel cells (Fig. 64), indicating that the overexpression of MITF is the driving element causing mTORC1 hyperactivity in these melanomas.

Fig. 64. Hyperproliferation of 501Mel cells is reduce upon depletion of MITF or RagD.

Proliferation index of 501Mel cells transfected with SCR, RagD, or MITF siRNAs. Plot represents means of three independent experiments ± SEM; one-way ANOVA (**p < 0.01, ***p < 0.001, 

Human tumor xenograft are a valuable model to investigate the contribute of a specific molecular alteration to tumor growth. Thus, to assess the contribute of RagD upregulation in malignancies associated with MiT/TFE deregulation, in collaboration with Prof. Pellicci, at the European Institute of Oncology (IEO) in Milan, we performed xenografts experiments by using 501Mel cells silenced for RagD or with control shRNA against luciferase. We observed a drastic reduction in tumor mass development upon silencing of RagD compared to the tumor mass originated from control 501Mel cells (Fig.65). These data clearly support an important role for RagD in promoting tumor growth in tumors dependent on MiT/TFE hyperactivation.

**Fig.65. Xenograft tumor growth is reduced in melanoma cells silenced for RagD.**

501Mel cells were infected with a lentivirus expressing a short hairpin RNA targeting the Luciferase (control, Sh-Luc) or RagD mRNAs and transplanted in NOD scid gamma (NSG) mice. **Left panel:** Representative picture of tumors isolated from both groups of mice. **Right panel:** Plot shows tumor volumes. Each dot represents a tumor. Twelve tumors (n = 12 mice) were analyzed per group; (**p < 0.001, Student’s t test).
DISCUSSION

The lysosome was initially discovered as a digestive organelle, designated to the degradation and recycling of cellular waste (de Duve, 2005). However, over the years, its role has been better dissected and the lysosome has emerged as a key metabolic signaling center for the cell, thanks to its ability to transport nutrients, sense their availability, and communicate this information to growth-regulatory pathways (Perera and Zoncu, 2016). mTORC1 signaling is the main regulatory pathway involved in metabolic adaptation to nutrient availability in the cell and it exerts its activity on the lysosomal surface. In presence of nutrients, mTORC1 is active on the lysosome and promotes growth and energy storage, thus activating anabolic pathways; whereas, when nutrients are missing, mTORC1 is inactive and therefore catabolic pathways are activated to provide the required building blocks for sustain cell survival (Laplante and Sabatini, 2012; Saxton and Sabatini, 2017). In this way, mTORC1 regulates the balance between biosynthetic and catabolic states. The new view of the lysosome as signaling platform has been supported by the identification of TFEB as the "master regulator of lysosomes", controlling the expression of genes involved in lysosomal biogenesis, exocytosis, and autophagy (Sardiello et al., 2009; Palmieri et al., 2011). TFEB represents the crucial node of a lysosome-to nucleus signaling mechanism through which lysosomal functions can be coordinated to respond and to adapt to environmental cues.

TFEB, TFE3 and MITF subcellular localization is regulated by mTORC1-phosphorylation: when nutrients are available, the MiT/TFE factors are phosphorylated and retained inactive into the cytoplasm, whereas in starvation condition, mTORC1 is inactive and they can translocate into the nucleus, activating their target genes (Martina et al., 2012, 2013, 2014; Roczniak-Ferguson et al., 2012; Settembre et al., 2011, 2012). mTORC1 inhibition of TFEB, and also
TFE3, activity is an additional way to block catabolic processes, triggering the transcriptional regulation of autophagic genes.

However, data from literature suggest that mTOR complexes may operate in the context of feedback loops, implying that downstream effectors are also likely to be involved in upstream regulation, to maintain various aspects of cellular homeostasis (Eltschinger et al., 2016). Thus, we reasoned that MiT/TFE members, as nutrient sensitive transcription factors, could in turn affect mTORC1 activity. Among the different inputs regulating mTORC1 signaling, amino acids levels are the most conserved ones, sensed through a complex machinery converging to the lysosome (Saxton and Sabatini, 2017). Upon amino acids stimulation, we observed that the levels of phosphorylation of mTORC1 substrates fluctuate together with TFEB expression levels. Moreover, TFEB overexpression is able to enhance common redouts of mTORC1 activity, such as cell proliferation and cell size. These were the first evidences that supported the existence of a negative feedback loop mechanism by which TFEB is itself able to regulate its inhibitor. The following question was to elucidate the detailed mechanism of action and TFEB target gene genes responsible for mTORC1 upregulation. We first hypothesized that autophagy was the link in between TFEB and mTORC1, since TFEB, as well as TFE3, are critical regulators of this process and, due to proteolysis events, autophagy is a source of amino acids in the lysosomal lumen. We thus evaluated whether these family of transcription factors regulate mTORC1 activity by modulating autophagy. However, we showed that TFEB-mediated activation of mTORC1 did not require the essential autophagy genes Atg5 or Atg7, suggesting an autophagy-independent mechanism. We then decided to dissect the members of mTORC1 pathway, looking for putative TFEB target gene genes. Starting from a bioinformatic analysis, looking for CLEAR elements in the promoters of mTORC1-
related genes, and subsequent validation experiments, *RagD* emerged as the putative target gene. RagD is one of the four RagGTPases present in mammals. The amino acid signaling centers around these proteins: in presence of amino acids, the active heterodimers RagA or B with RagC or D, promote mTORC1 recruitment on the lysosomal surface, which is necessary for its activation by Rheb (Kim *et al.*, 2008; Sancak *et al.*, 2008). The role of these GTPases has emerged during the last decade and this field is getting more and more complex over time.

A recent paper by Lawrence *et al.* underlines their dynamic functions, claiming that the RagGTPases cycles between the lysosome and the cytoplasm in order to tightly regulate mTORC1 interaction with Rheb on the lysosome, thus its signaling. As for our work, we then analysed mTORC1 recruitment on the lysosome and how this could be affected by TFEB levels. Supporting the existence of a TFEB-RagD-mTORC1 axis, we observed that mTORC1 lysosomal localization is increased when TFEB is overexpressed. These data supported our hypothesis of this axis as a rapid way by which TFEB could rapidly switch off itself, increasing the amount of mTORC1 on the lysosome ready to be reactivated by nutrients. Indeed, we found a significant correlation between starvation-induced TFEB nuclear localization and RagD expression levels, at both mRNA and protein levels. Studies in mice confirmed RagD induction in fasting and TFEB upregulation of mTORC1 signaling, introducing the new mechanism identified in a physiological context. We then investigated more in detail the role of TFEB-RagD-mTORC1 axis in specific tissues that are remarkably influenced by mTORC1 activity. The liver is a metabolic organ particularly sensitive to nutrients, growth factors and energy, and thereby plays a central role in carbohydrate, protein, amino acids, and lipid metabolism. In Ballabio’s group, we had available *Tcfeb*-liver specific conditional KO mice and we confirmed the impaired mTORC1 signaling in the livers of these
mice. The reduction in the protein synthesis rate in Tcfeb-LiKO mice is another evidence of the role of TFEB in cellular adaptation to nutrient availability. Moreover, up to date, it's widely known that mTORC1 promotes protein synthesis in muscles upon physical exercise to support muscle growth. This effect is well appreciated by bodybuilders, who know that eating a protein-rich meal after exercise is a good strategy to increase muscles. However, the mechanisms underlying this process are still poorly understood. Interestingly, TFEB nuclear translocation is induced upon physical exercise as a consequence of its phosphatase activation (Medina et al., 2015). Therefore, we hypothesized that TFEB could regulate mTORC1 signaling in response to exercise. Indeed, we observed that exercised muscle-specific Tcfeb KO mice showed a reduced induction of mTORC1 activity and protein synthesis in response to leucine after exercise, indicating that mTORC1 activation upon physical exercise also requires MiT/TFE factors. Hence, our findings suggested the existence of a new molecular pathway, physiologically relevant, that collocated mTORC1 downstream of MiT/TFE factors.

How modulation of RagD levels alone can be sufficient to significantly impair mTORC1 signaling remains an open question. One would expect that RagC could compensate for RagD but our study suggest that these two Rags are less interchangeable than what was previously thought. Another critical point is that to be functionally active RagD needs to form heterodimers with either RagA or B. We noticed that RagD levels are lower compared to the other Rag GTPases and therefore, one possibility is that RagD represents a limiting factor for RagD/RagA and RagD/RagB heterodimer formation. In line with this hypothesis is the observation that perturbing MiT/TFE mediated transcriptional regulation of RagD only is sufficient to severely hampers mTORC1 lysosomal recruitment and hence
its activation. In summary, we defined a new regulation of mTORC1 activity in response to nutrients. Starvation inhibits mTORC1 thus allowing MiT/TFE nuclear translocation and hence activation of catabolism via the expression of autophagic and lysosomal genes. In the meantime, MiT/TFE factors induce the expression of RagD and this results in the assembly of inactive Rags heterodimers on the lysosomal surface. Feeding turn on Rags, which can now recruit mTORC1 to the lysosome and promote its activation. In this way, the cell gets ready to efficiently switch on anabolism and turn off catabolism when nutrients become available.

Once identified the TFEB-RagD-mTORC1 axis and its physiological relevance, we asked whether this axis could be implicated in pathological conditions. Due to the reported role in cancer of both the MiT/TFE factors, described as oncogenes in several type of cancers (Haq and Fisher, 2011; Kaufmann et al., 2014; Perera et al., 2015), and of mTORC1, highly implicated in cancer pathogenesis, mainly fueling tumor growth (Laplante and Sabatini, 2012), we investigated if the MiT/TFE-RagD-mTORC1 axis was deregulated in MiT/TFE-overexpressing tumors. The characterization of Renal Cell Carcinoma models, overexpressing TFEB or TFE3, and of patients-derived melanoma cells, overexpressing MITF, showed the increased activity of mTORC1, due to MiT/TFE overexpression, and upregulation of RagD. Xenografts experiments highlighted the role of the MiT/TFE-RagD-mTORC1 deregulated axis in tumorigenesis, confirming the involvement of this mechanism in tumor growth. These findings define a new oncogenic pathway in MiT/TFE dependent tumors with potential implications for therapy. Defining additional interacting partners and transcriptional targets of MiT/TFE proteins and further characterization of upstream signaling cascades that control MiT/TFE levels, stability, localization, and activity may contribute to find novel therapeutic strategies to switch off MiT/TFE in cancer cells.


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