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Modulating TFEB activity in the Birt-Hogg-Dubé syndrome by targeting the vacuolar ATPase

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LIST OF ABBREVIATIONS

4EBP1	Eukaryotic translation initiation factor 4E Binding Protein 1
ACC	Acetyl-CoA Carboxylase
AD	Activation Domain
AMPK	5' AMP-activated protein Kinase
ARF1/6	ADP Ribosylation Factor 1 and 6
ARNO	ARF Nucleotide-binding site Opener
ASPS	Alveolar Soft Part Sarcoma
ATF4	Activating Transcription Factor 4
ATG101	Autophagy related 101
ATG16L1	Autophagy related 16 Like 1
ATP	Adenosine Triphosphate
ATP6AP1/2	ATPase H^+ transporting Accessory Protein 1 and 2
ATP6V0C	ATPase H^+ transporting V0 subunit c
ATP6V0D2	ATPase H^+ transporting V0 subunit d 2
ATP6V1A	ATPase H^+ transporting V1 subunit A
ATP6V1D	ATPase H^+ transporting V1 subunit D
BafA1	Bafilomycin A1
BHD	Birt-Hogg-Dubé
bHLH-LZ	Basic Helix-Loop-Helix Leucine-Zipper
C/EBP-α	CCAAT Enhancer Binding Protein alpha
CAD	Carbamoyl-phosphate synthetase 2, Aspartate transcarbamylase, and Dihydroorotase
cAMP	Cyclic Adenosine Monophosphate
CASTOR	Cellular Arginine Sensor for mTORC1
CDK4/6	Cyclin Dependent Kinase 4 and 6
CLEAR	Coordinated Lysosomal Expression And Regulation motif
ConA	Concanamycin A

CQ	Chloroquine
CTRL	Control
DEPDC5	DEP Domain-Containing 5
DEPTOR	DEP domain containing mTOR interacting protein
dKO	Double Kock Out
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EGF	Epidermal Growth Factor
ERK1/2	Extracellular signal-Regulated Kinase 1 and 2
FASN	Fatty Acid Synthase
FIP200	Focal adhesion kinase family Interacting Protein of 200kDa
FLCN	Folliculin
FNIP1/2	Folliculin-Interacting Protein 1 and 2
GAP	GTPase Activating Protein
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GATOR1/2	GAP activity toward Rags complex 1 and 2
GCN5	General Control Non-repressed 5
GDP	Guanosine Diphopshate
GEF	Guanine nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GSK3β	Glycogen Synthase Kinase 3 beta
GST	Glutathione-S-Transferase
GTP	Guanosine Triphosphate
HA	Human influenza Hemagglutinin
HCS	High Content Screening
HDAC6	Histone Deacetylase 6
HK-2	Human Kidney 2
HOPS	Homotypic Fusion and Protein Sorting
HPRT1	Hypoxanthine Phosphoribosyl-Transferase 1

KICSTOR KPTN, ITFG2, C12orf66 and SZT2-containing regulator of TOR

KO Knock Out

l.e. Long Exposure

LAMP1 Lysosomal-Associated Membrane Protein 1

LAMTOR1-5 Late endosomal/lysosomal adaptor, MAPK and MTOR activator 1-5

LARS Leucyl-tRNA synthetase

LKB1 Liver Kinase B 1

LLOME L-Leucyl-L-Leucine Methyl Ester

LOH Loss Of Heterozygosity

LPS Lipopolysaccharide

LRRK2 Leucine Rich Repeat Kinase 2

LSDs Lysosomal Storage Disorders

mCherry Monomeric Cherry

MCOLN1 Mucolipin1 or TRPML1

MiT/TFE Microphthalmia family of transcription factors

MITF Microphthalmia-associated Transcription Factor

mLST8 Mammalian lethal with sec-13 protein 8

mSin1 Mammalian Stress-activated map kinase-Interacting protein 1

MTHFD2 Methylenetetrahydrofolate Dehydrogenase ($NADP^+$ dependent) 2

mTOR Mechanistic Target of Rapamycin

mTORC1 Mechanistic Target of Rapamycin kinase Complex 1

mTORC2 Mechanistic Target of Rapamycin kinase Complex 2

NES Nuclear Export Signal

NLS Nuclear Localization Signal

NPC1 Niemann-Pick C1

NPRL2/3 Nitrogen Permease Related-Like 2 and 3

ns Not Significant

PI3K Phosphatidylinositol 3-Kinase

PKA Protein Kinase A

PKB/AKT	Protein Kinase B or AKT
PKCα	Protein Kinase C Alpha
PKCβ	Protein Kinase C Beta
PKD	Protein Kinase D
PP2A	Protein Phosphatase 2A
PPAR-γ	Peroxisome Proliferator-Activated Receptor gamma
PRAS40	Proline-Rich Akt Substrate 40kDa
Protor1/2	Protein observed with Rictor 1 and 2
PTMs	Post Translational Modifications
Rags	Rag GTPases
RAPTOR	Regulatory-Associated Protein of mTOR
RBR	Rag-Binding Region
RCC	Renal Cell Carcinoma
RHEB	Ras Homolog Enriched in Brain
RICTOR	Rapamycin-Insensitive Companion of mTOR
RSK	p90 Ribosomal S6 Kinase
S6K	p70S6 Kinase
SaliPhe	Saliphenylhalamide
SAM	S-adenosylmethionine
SAMTOR	SAM sensor for mTOR
SCD-1	Stearoyl-CoA Desaturase 1
Scr	Scramble
SESN2	Sestrin2
SGK1	Serum/Glucocorticoid regulated Kinase 1
siRNA	Small Interfering RNA
SLC38A9	Solute Carrier family 38 Member 9
SREBP-1	Sterol Regulatory Element Binding Protein 1
STAT3	Signal Transducer and Activator Of Transcription 3
TBC1D7	TBC1 Domain family member 7

Tel2	Telomere Maintenance 2
TFE3	Transcription Factor E3
TFEB	Transcription Factor EB
TFEC	Transcription Factor EC
TIF-1A	Transcription Initiation Factor 1A
TOS	TOR signaling motif
TRPML1	Transient Receptor Potential Mucolipin 1
TSC	Tuberous Sclerosis Complex
TSC1/2	Tuberous Sclerosis Complex 1 and 2
Tti1	TELO2 interacting protein 1
UBF	Upstream Binding Factor
ULK1	Unc-51-Like autophagy-activating Kinase1
UVRAG	UV radiation Resistance-Associated Gene product
V-ATPase	Vacuolar (H^+)-ATPase
VEGF	Vascular Endothelial Growth Factor
WT	Wild-Type

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ABSTRACT

The transcription factor EB (TFEB) is a master regulator of lysosome biogenesis and autophagy and its activity is primarily controlled by the kinase complex mTORC1 which phosphorylates TFEB at conserved serine residues promoting its cytoplasmic localization. Several studies demonstrated that TFEB is constitutively nuclear and active in cellular and murine models of Birt Hogg Dubé (BHD) syndrome, a genetic disease caused by germline mutations in *FLCN* gene, and this promotes the development of kidney cysts and renal cell carcinomas associated with this condition. Therefore, inhibition of TFEB activity represents a challenging opportunity for the treatment of the BHD syndrome.

To this purpose, we performed a high content siRNA screening to identify correctors of TFEB nuclear localization in FLCN-KO cells. Top hits were genes encoding subunits of the vacuolar ATPase (V-ATPase), in particular ATP6V0C and ATP6V1A. The V-ATPase is a proton-pump protein complex responsible for acidifying and maintaining the pH of the lysosomes, and it has been described as a positive regulator of mTORC1 signaling, although the exact contribute of the V-ATPase to the amino acid sensing has not been fully elucidated. Our results indicate that the V-ATPase inhibition promotes a mTORC1-dependent TFEB cytosolic re-localization in FLCN-KO cells, thus pointing to a novel mechanism of regulation of TFEB activity in BHD cellular models mediated by the V-ATPase. Besides, our data suggest that the Ragulator-Rag GTPases complex plays a crucial role in controlling TFEB subcellular localization, even in absence of FLCN, through its interaction with the V-ATPase.

This study aims at dissecting how the V-ATPase regulates mTORC1 signaling and TFEB activity, while searching for compounds able to target this axis, with the purpose to identify novel and effective therapeutic approaches to limit kidney pathologies associated with BHD syndrome.

INTRODUCTION

1. MiT/TFE transcription factor family

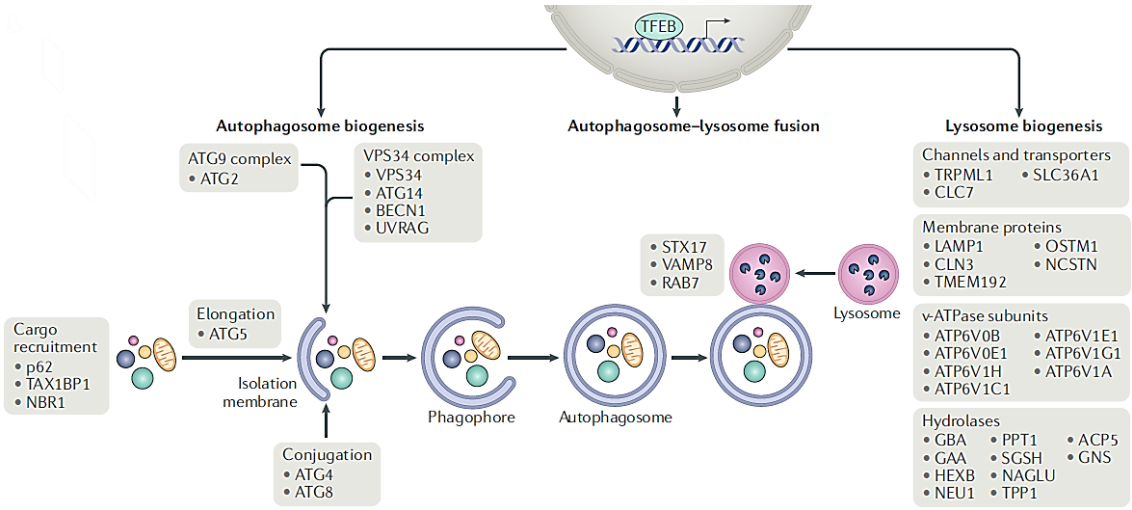
The microphthalmia transcription factors (MiT/TFE) belong to the basic helix-loop-helix leucine zipper (bHLH-LZ) family of proteins. In vertebrates, the MiT/TFE family is composed of four evolutionarily conserved and closely related members: microphthalmia-associated transcription factor (MITF), transcription factor EB (TFEB), transcription factor E3 (TFE3), and transcription factor EC (TFEC) (Steingrímsson et al. 2004). These transcription factors are key regulators of cellular homeostasis, particularly in processes related to cell differentiation, development, metabolism and response to environmental cues (La Spina *et al.*, 2020). MiT/TFE factors, like any other bHLH-LZ factors, form homodimers and heterodimers that bind a hexanucleotide palindromic CACGTG sequence, called E-box, in the proximal promoter of target genes (Hemesath *et al.*, 1994). MiT/TFE proteins also specifically recognize the asymmetric TCATGTG M-box response elements present in the promoter region of their target genes (Aksan and Goding, 1998). Finally, TFEB and TFE3 also directly bind the CLEAR (Coordinated Lysosomal Expression And Regulation) element, a palindromic 10-base pair motif (GTCACGTGAC) that is present in many lysosomal genes (Sardiello *et al.*, 2009).

1.1. Transcription factor EB (TFEB)

TFEB is a key regulator of cellular homeostasis by controlling lysosomal biogenesis and autophagy, two essential cellular processes involved in the degradation and recycling of cellular components (Napolitano and Ballabio, 2016). TFEB directly binds the CLEAR motif in the promoter of genes involved in lysosomal biogenesis and function, including lysosomal enzymes, lysosomal transmembrane proteins and several subunits of the v-ATPase (Fig.1) (Sardiello *et al.*, 2009; Palmieri *et al.*, 2011). Additionally, TFEB activity is involved also in the regulation of other lysosome-associated processes, including autophagy, exocytosis and endocytosis,

release of intracellular Ca^{2+}, phagocytosis and immune response (Settembre *et al.*, 2011; Nnah *et al.*, 2019; Franco-Juárez *et al.*, 2022). However, TFEB transcriptional program is not limited to lysosome homeostasis and activity, but it regulates function and degradation of other cellular compartments, including endoplasmic reticulum and mitochondria (Kim *et al.*, 2018; Cinque *et al.*, 2020). Interestingly, TFE3 governs organelles biogenesis and the process of autophagy by regulating a gene network that significantly intersects with the network controlled by TFEB (Martina *et al.*, 2014).

Hence, through its influence on autophagy, lysosomal biogenesis and exocytosis, TFEB coordinates a transcriptional program that governs fundamental cellular degradative pathways and promotes clearance of intracellular substrates.



Adapted from Ballabio and Bonifacino, Nat Rev Mol Cell Biol, 2020

Figure 1. TFEB transcriptional regulation of the lysosomal–autophagic pathway. TFEB regulates genes involved in several steps of the lysosomal–autophagic pathway, including cargo recruitment, autophagosomes biogenesis, autophagosome–lysosome fusion and lysosomal biogenesis.

1.2. Regulation of TFEB activity

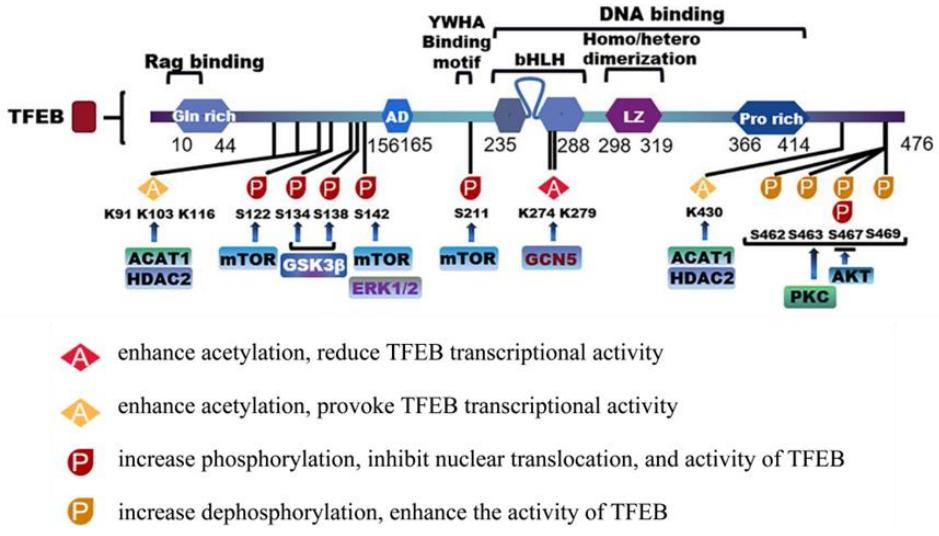
TFEB responds to a variety of environmental cues that modulate its subcellular localization. In normal and resting condition, TFEB is cytosolic and inactive, while under cellular stress or energy restriction, TFEB translocates to the nucleus where

it promotes the transcription of its target genes (Li *et al.*, 2018; Napolitano *et al.*, 2018). The subcellular localization and activity of TFEB are strictly regulated through post-translational modifications (PTMs) and protein–protein interactions.

The principal mechanism that causes TFEB cytosolic sequestration is the phosphorylation of specific serine residues. Different kinases are responsible for TFEB phosphorylation on different residues, including mTOR, AMPK, ERK2, GSK3, AKT, PKCβ and CDK4/6 (Fig.2) (Puertollano *et al.*, 2018; Franco-Juárez *et al.*, 2022). On the other hand, the phosphatases Calcineurin and Protein Phosphatase 2 (PP2A) de-phosphorylate TFEB in response to lysosomal calcium release and under oxidative stress, respectively (Medina *et al.*, 2015; Martina and Puertollano, 2018). Among all phosphosites, two particular serine residues, targeted by mTOR and ERK2, are fundamental in determining TFEB subcellular localization: Ser142 is localized near a nuclear export signal (NES), implying a possible role for TFEB nuclear exclusion (Settembre *et al.*, 2011, 2012; Li *et al.*, 2018); phosphorylated Ser211 is required for TFEB binding with the chaperone 14-3-3, which allows cytosolic retention by masking a nuclear localization signal (NLS) (Martina *et al.*, 2012). Under nutrient-rich conditions, phosphorylation of both serine 142 and 211 keeps TFEB inactive in the cytosol. Consistently, TFEB variants carrying Serine-to-Alanine mutations of these serine residues are always nuclear and constitutively active (Settembre *et al.*, 2011; Martina *et al.*, 2012). Inversely, nutrient deprivation induces TFEB de-phosphorylation by Calcineurin, a phosphatase that is activated by release of lysosomal calcium through the Ca^{2+} channel mucolipin 1 (MCOLN1 or TRPML1) (Medina *et al.*, 2015). This mechanism is central for TFEB activation, since depletion of MCOLN1 impairs TFEB nuclear translocation.

Interestingly, the mechanisms governing TFEB regulation is also responsible for controlling the activity of the other MiT/TFE factors. For instance, TFE3 and some isoforms of MITF undergo phosphorylation by mTORC1 on similar conserved serine residues and exhibit similar nucleo-cytoplasmic shuttling properties (Martina *et al.*, 2014; Martina and Puertollano, 2018).

In recent years, increasing interest is arising for other PTMs that control TFEB activity. Several studies suggested that acetylation of specific lysine residues is required for TFEB nuclear transport and activity (Fig.2). Specifically, deacetylated mimic mutations of lysine 91, 103, 116 and 430 to arginine decrease TFEB nuclear accumulation and transcriptional activity; accordingly, histone deacetylation inhibitors activate autophagy through a TFEB-dependent mechanism (Zhang *et al.*, 2018). In addition, the histone deacetylase 6 (HDAC6) can target TFEB and detain it in the cytosol (Brijmohan *et al.*, 2018). Conversely, acetylation on Lys116, 247 and 279 by the histone and lysine acetyltransferase GCN5 impairs TFEB dimerization and its binding to target gene (Wang *et al.*, 2020). These data suggest that TFEB acetylation pattern affects its activation, but whether this PTM promotes or not TFEB activity is still unclear.



Adapted from Li M. et al., Front Physiol, 2021

Figure 2. TFEB structure and its phosphorylation and acetylation sites. TFEB sequence includes a glutamine-rich (Gln rich) region, an acidic transcription activation domain (AD), the basic helix-loop-helix leucine-zipper (bHLH-LZ) structure, and proline-rich (Pro rich) motifs. TFEB activity is modulated by phosphorylation and acetylation. The figure shows TFEB phosphorylated or acetylated sites and the enzymes targeting these residues.

Besides phosphorylation and acetylation, TFEB has been shown to undergo PARsylation, SUMOylation, glycosylation, and cysteine oxidation (Li *et al.*, 2021;

Franco-Juárez *et al.*, 2022), but the mechanisms that regulate these PTMs and their effect on TFEB activity are still controversial and require more in-depth studies.

The fine regulation of TFEB by different PTMs highlights the relevance of this transcription factor in orchestrating different pathways essential for cellular survival.

1.3. Physiological roles of TFEB

By regulating the transcriptional expression of several genes, TFEB displays different tissue-specific roles.

During embryo development, TFEB activity is important for placental vascularization, since TFEB-null mice die at embryonic day (E)9.5–10.5 because of defective VEGF expression (Steingrímsson *et al.*, 1998). Therefore, generation of tissue-specific conditional knock-out mouse models turned out highly advantageous in elucidating TFEB's role within various tissues and organs.

Specific TFEB deletion in the liver results in severe obesity due to impaired lipid metabolism (Settembre, De Cegli, *et al.*, 2013; Pastore *et al.*, 2017).

In skeletal muscle, TFEB-mediated modulation of glucose uptake and mitochondrial biogenesis is required during physical exercise (Mansueto *et al.*, 2017).

TFEB-dependent lysosomal gene expression is necessary for bone resorption by osteoclasts, indeed TFEB depletion impairs osteoclast function and increase bone mass (Ferron *et al.*, 2013).

In the intestinal epithelium, deletion of TFEB causes cell damage and colitis (Murano *et al.*, 2017), revealing a protective role of TFEB in preventing tissue injury.

Moreover, TFEB stimulates angiogenesis through activation of AMPK and autophagy in endothelial cells (Fan *et al.*, 2018).

Several studies have described TFEB as a modulator of both innate and adaptative immune response. In particular, loss of both TFEB and TFE3 in macrophages impairs production and secretion of some pro-inflammatory cytokines and chemokines (Pastore *et al.*, 2016). TFEB also stimulates antigen presentation by dendritic cells

and T cell-dependent antibody response (Huan *et al.*, 2006; Samie and Cresswell, 2015). Furthermore, pathogens infection activates TFEB via the protein kinase D (PKD) pathway, and induce transcriptional expression of several antimicrobial and autophagic genes (Visvikis *et al.*, 2014; Najibi *et al.*, 2016).

Finally, TFEB and TFE3 were found to participate to circadian cycles by regulating the whole-body metabolism and autophagy during the diurnal light (Pastore *et al.*, 2019).

Anyway, TFEB is entangled in a number of other physiological processes, including cardioprotection, neuronal survival and aging (Santin *et al.*, 2016; Su *et al.*, 2018; Abokyi, Ghartey-Kwansah and Tse, 2023). Elucidating TFEB regulatory pathways therefore represents a critical goal for human health preservation.

1.4. The role of TFEB in human diseases

MiT/TFE factors were found to be dysregulated in many diseases. Remarkably, TFEB overexpression can be both a protective or pathological factor.

In Lysosomal storage diseases (LSDs), a group of disorders caused mainly by enzyme deficiencies within the lysosome, TFEB overexpression induces cellular clearance of undigested materials accumulated in lysosomes, thus ameliorating the disease phenotype (Medina *et al.*, 2011; Song *et al.*, 2013; Spampanato *et al.*, 2013). In neurodegenerative disorders such as Alzheimer's (Polito *et al.*, 2014; Xiao *et al.*, 2014; Chauhan *et al.*, 2015), Parkinson's (Decressac and Björklund, 2013; Kilpatrick *et al.*, 2015; Arotcarena *et al.*, 2019) and Huntington's diseases (Tsunemi *et al.*, 2012; Vodicka *et al.*, no date), TFEB induction prevents protein aggregates formation and accumulation. Furthermore, liver pathologies, such as α1-antitrypsin deficiency and obesity, benefit from TFEB upregulation, resulting in enhanced autophagy or lipophagy, respectively (Pastore *et al.*, 2013; Settembre, Fraldi, *et al.*, 2013).

In contrast, TFEB and the other MiT/TFE members of transcription factors are known oncogenes that promote tumorigenesis and regulate energy metabolism in different

types of cancer (Zoncu and Perera, 2023). By promoting autophagy, TFEB can support tumor cells by providing nutrients and energy during periods of stress, such as limited nutrient availability and chemotherapy (Perera, Di Malta and Ballabio, 2019). TFEB and TFE3 translocations and rearrangements were found in pediatric renal cell carcinoma (RCC) and alveolar soft part sarcoma (ASPS) (Argani *et al.*, 2001; Ramphal *et al.*, 2006). In kidney cancers associated with translocations of *TFE* genes, TFEB overexpression induces the canonical WNT signaling, a key pathway implicated in tumor pathogenesis (Calcagnì *et al.*, 2016). TFEB dysregulation was detected also in non-small cell lung cancer (Giatromanolaki *et al.*, 2015), prostate cancer (Zhu *et al.*, 2021), colorectal cancer (Liang *et al.*, 2018) and pancreatic cancer (Kim *et al.*, 2021). Notably, TFEB knockdown or inhibition can sensitize resistant cancer cells to chemotherapy, by modulating DNA-damage response, apoptosis, autophagy and lysosomal activity (Zhitomirsky and Assaraf, 2015; Slade *et al.*, 2020; Kao *et al.*, no date).

Finally, TFEB and TFE3 constitutive activation is a main driver of disease phenotype of two inherited cancer syndromes, the Birt-Hogg-Dubé (BHD) syndrome and the Tuberous Sclerosis Complex (TSC), autosomal dominant diseases characterized by high risk for developing multiple types of tumors (Napolitano *et al.*, 2020; Alesi *et al.*, 2021; Di Malta *et al.*, 2023).

Together these studies suggest that modulation of TFEB could represent a new therapeutic strategy for a broad variety of diseases.

2. The mechanistic target of rapamycin (mTOR)

The mechanistic Target of Rapamycin (mTOR) is a serine/threonine kinase belonging to the phosphoinositide 3-kinase (PI3K)-related family of protein kinases that integrates multiple environmental and intracellular signals to modulate cell growth and metabolism. The mammalian mTOR and its homologue TOR in yeast were identified as targets of Rapamycin, a compound produced by *Streptomyces Hygroscopius* bacteria that inhibits cell growth and proliferation (Heitman, Movva

and Hall, 1991; Kunz *et al.*, 1993; Sabatini *et al.*, 1994). Given mTOR central role in nutrient sensing, energy balance and autophagy, it is not surprising that mTOR pathways were found dysregulated in different diseases, including cancer (Laplante and Sabatini, 2012).

2.1. The mTOR complexes: mTORC1 and mTORC2

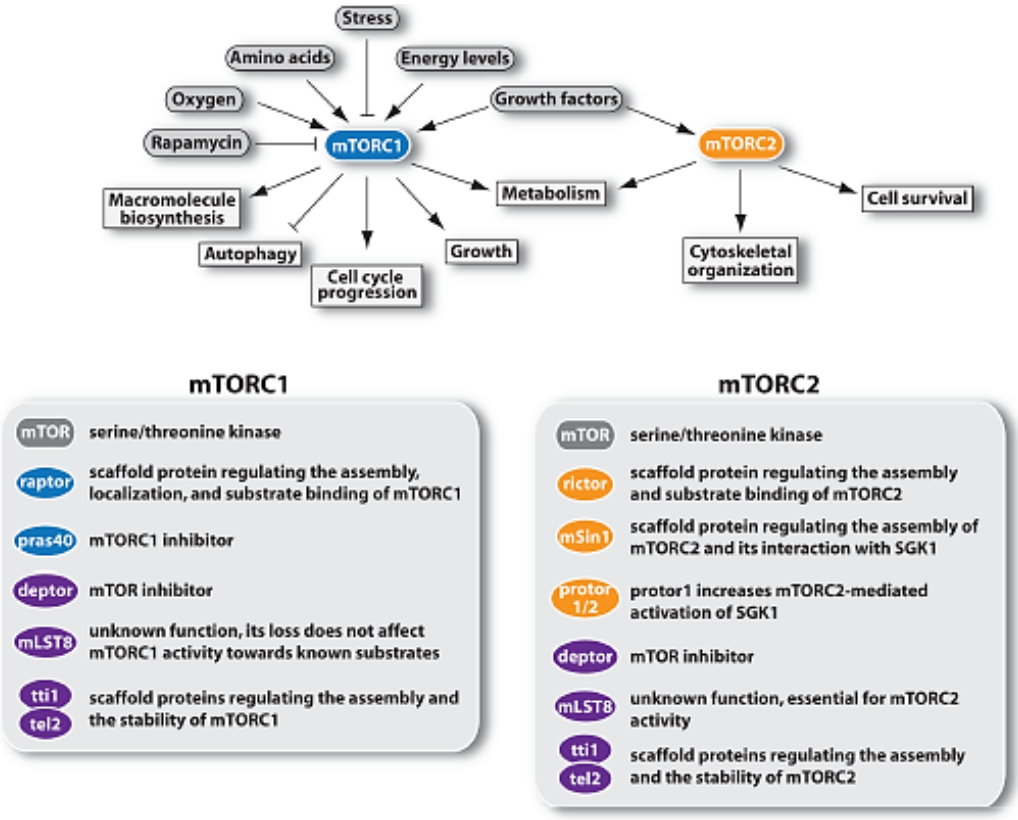
mTOR acts as a catalytic subunit of two different multi-subunit complexes, mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2), distinguishable by unique accessory proteins. These complexes share the mTOR kinase, mLST8 (mammalian lethal with SEC13 protein 8, also known as GβL), Deptor (DEP domain-containing mTOR-interacting protein), and the Tti1/Tel2 (Kim *et al.*, 2003; Peterson *et al.*, 2009; Kaizuka *et al.*, 2010). In contrast, the mTORC1 includes Raptor (regulatory-associated protein of mTOR) and PRAS40 (proline-rich Akt substrate 40kDa) (Hara *et al.*, 2002; Kim *et al.*, 2002; Oshiro *et al.*, 2007; Vander Haar *et al.*, 2007; Wang *et al.*, 2007), while mTORC2 comprises Rictor (rapamycin-insensitive companion of mTOR), mSin1 (mammalian stress-activated map kinase-interacting protein 1), and Protor1/2 (protein observed with Rictor 1 and 2) (Sarbassov *et al.*, 2004; Frias *et al.*, 2006; Jacinto *et al.*, 2006; Pearce *et al.*, 2007). The two mTOR complexes exhibit different sensitivities to rapamycin, as well as differences in their responses to upstream signals and downstream effects. Figure 3 summarizes components and functions of mTORC1 and mTORC2.

In the mTOR complexes, mLST8 stabilizes mTOR kinase activation loop and can stimulates its activity (Kim *et al.*, 2003; Yang *et al.*, 2013), while Deptor negatively regulates mTOR (Peterson *et al.*, 2009). Eventually, Tti1 and Tel2 are required for correct complexes assembly (Kaizuka *et al.*, 2010).

In the mTORC1 complex, Raptor is required for recruitment of mTORC1 substrates through binding to the TOR signaling (TOS) motif, a consensus sequence of five amino acid discovered in some mTORC1 targets (Hara *et al.*, 2002; Nojima *et al.*,

2003; Schalm *et al.*, 2003). Conversely, PRAS40 exerts an inhibitory function towards mTOR (Vander Haar *et al.*, 2007; Wang *et al.*, 2007).

In the mTORC2 complex, the other components Rictor, mSIN1 and Protor1/2 stabilize the assembly of the complex, and they also participate to phosphorylation of some substrates by mTORC2, including protein kinase B (PKB/AKT), protein kinase C alpha (PKCα) and the serin/threonine-protein kinase SGK1 (Sarbassov *et al.*, 2004, 2005; Frias *et al.*, 2006; Guertin *et al.*, 2006; Lu *et al.*, 2011; Pearce *et al.*, 2011).



Adapted from Laplante and Sabatini, Cell, 2012

Figure 3. Overview of mTORC1 and mTORC2 regulatory inputs, functions and components. The mTOR kinase can form two distinct protein complexes known as mTORC1 and mTORC2. While mTORC1 is highly responsive to various signals including amino acids, stress, energy availability, and growth factors, mTORC2 responds primarily to growth factors. Moreover, unlike mTORC1, mTORC2 is not acutely affected by rapamycin. The bottom panel of the figure schematizes the functions of the individual protein components that constitute the mTOR complexes.

mTORC1 and 2 are activated by distinct upstream stimuli. In particular, mTORC1 responds to amino acids, glucose, growth factors, energy and stress to modulate cell growth and metabolism. On the other hand, mTORC2 mainly regulates cell proliferation and survival in response to growth factors and hormones (Laplante and Sabatini, 2012; Saxton and Sabatini, 2017).

The two mTOR complexes also differ in their sensitivity to drug inhibitors. For instance, catalytic inhibitors of mTOR (e.g. Torin1) completely inhibit both complexes, but only mTORC1 is sensitive to treatment with Rapamycin (Sarbassov *et al.*, 2004; Ballou and Lin, 2008; Feldman *et al.*, 2009).

2.2. Regulation of mTORC1

In nutrient-rich conditions, mTORC1 is activated and stimulates anabolic processes such as synthesis of protein, lipids and nucleotides. Simultaneously, mTORC1 inhibits catabolic processes such as autophagy (Liu and Sabatini, 2020). Conversely, during fasting the degradative catabolic pathways are favored over the anabolic ones, thus mTORC1 is inhibited. mTORC1 activation requires two steps: its translocation to the lysosome and stimulation of its kinase activity.

mTORC1 can be activated by several intracellular and extracellular signals via different signaling pathways (Fig.4). Growth factors, glucose, energy supply and insulin modulate mTORC1 activity through the tuberous sclerosis complex (TSC), an heteromeric signaling node composed of TSC1, TSC2 and TBC1D7 (TBC1 Domain Family Member 7) (Dibble *et al.*, 2012). TSC is a negative regulator of mTORC1 that acts as a GTPase activating protein (GAP) for Rheb (Ras homolog enriched in brain), an activator of mTOR kinase activity (Inoki, Zhu and Guan, 2003; Tee *et al.*, 2003). In basal condition, when nutrients are present and growth factors promote cell proliferation, TSC is phosphorylated and inhibited by AKT, ERK1/2 or RSK (p90 ribosomal S6 kinase), causing the dissociation of TSC from the lysosomal surface and relieve inhibition of Rheb (Inoki *et al.*, 2002; Harrington *et al.*, 2004; Ma *et al.*, 2005). Vice versa, under cellular stress, nutrient withdrawal, energy and oxygen

deficiency, TSC is phosphorylated and activated by AMPK (5' AMP-activated protein kinase) and GSK3β (the Glycogen synthase kinase-3 beta), leading to the conversion of Rheb from the active GTP-loaded state to the inactive GDP-bound state. The AMPK signaling pathway is a catabolism enhancer that is triggered by low cellular ATP levels, and leads to mTORC1 inhibition by phosphorylating Raptor and by activating TSC (Inoki, Zhu and Guan, 2003; Gwinn *et al.*, 2008).

Independently of TSC, AKT activation by growth factors also favors mTORC1 activity through phosphorylation of PRAS40, an endogenous inhibitor of mTORC1, causing its sequestration by the cytosolic anchor protein 14-3-3 (Vander Haar *et al.*, 2007). Although the mTORC1 pathway is a metabolic hub that responds to several inputs, amino acids play a major role in modulating mTORC1 activity. In particular, amino acids directly controls mTORC1 recruitment to the lysosome through the Ragulator-Rag GTPases complex (Kim *et al.*, 2008; Sancak *et al.*, 2010). Ragulator is a pentameric complex composed of p18, p14, MP1, C7orf59 and HBXIP, also known as Lamtor1–Lamtor5 (Late Endosomal/Lysosomal Adaptor, MAPK And MTOR Activator 1-5) (Sancak *et al.*, 2010; Zhang *et al.*, 2017). Lamtor1 is responsible for anchoring the complex to the lysosomal surface by its myristoylated and palmitoylated N-terminus (Nada *et al.*, 2009; de Araujo *et al.*, 2017). The Ragulator complex interacts with Rag GTPase, acting as a scaffold on the lysosomal membrane. Rag GTPases (Rags) are four guanosine triphosphatases that form obligate heterodimers composed of RagA/B associated with RagC/D (Kim *et al.*, 2008; Sancak *et al.*, 2008). RagA/B dimer is active when GTP-loaded, while RagC/D is active when it binds GDP. Once activated by amino acids (RagA/B^{GTP}-RagC/D^{GDP}), the Rags bind Raptor, thus recruiting mTORC1 to the lysosome and allowing mTOR activation by Rheb (Sancak *et al.*, 2008, 2010). Conversely, amino acid withdrawal locks the Rags in an inactive state (RagA/B^{GDP}-RagC/D^{GTP}) that is not able to interact with mTORC1, thereby causing its inactivation. Rag GTPases nucleotide-binding state is modulated by intersubunit communication between the Rags (Shen, Choe and Sabatini, 2017) and by several amino acid-sensitive guanine nucleotide

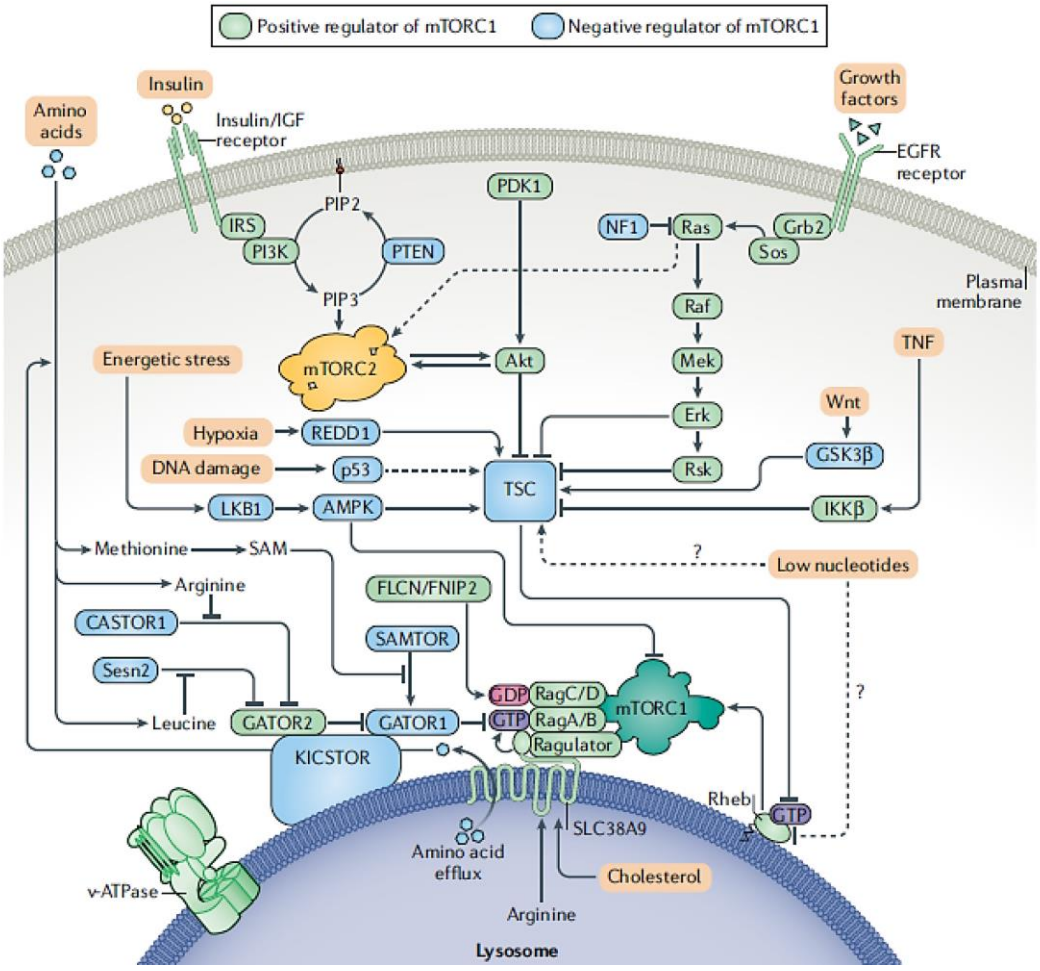
exchange factors (GEFs) and GTPase-activating proteins (GAPs). In absence of amino acids, RagA/B are inhibited by GATOR1 (GAP activity toward Rags complex 1), a trimeric complex consisting of DEPDC5 (DEP domain-containing 5), NPRL2 and NPRL3 (nitrogen permease related-like 2 and 3) (Bar-Peled *et al.*, 2013). GATOR1 exerts its GAP activity towards RagA/B on the lysosomal surface, where it is recruited by the KICSTOR complex (KPTN-, ITFG2-, C12orf66-, and SZT2-containing regulator of TOR) (Wolfson *et al.*, 2017). Furthermore, GATOR1 is inhibited by GATOR2, a pentameric complex composed of WDR24, WDR59, MIOS, SEH1L and SEC13 (Bar-Peled *et al.*, 2013). Availability of amino acids is conveyed to the Rags via the action of dedicated sensors that modulate GATOR complexes. For instance, Sestrin (SESN2) and CASTOR (cellular arginine sensor for mTORC) respectively sense cytosolic leucine and arginine abundance, and inhibit GATOR2 upon amino acid starvation. On the other hand, deprivation of methionine or the S-adenosylmethionine (SAM), a byproduct derived from methionine, stimulates SAMTOR, a SAM sensor that negatively regulates mTORC1 by binding with GATOR1 and KICSTOR (Gu *et al.*, 2017).

In addition to GATOR complexes, amino acids availability is also sensed by the FLCN (folliculin)-FNIP1/2 (folliculin interacting protein 1/2) complex. FLCN is a GAP for RagC/D, thus it promotes their activation when amino acids are present (Tsun *et al.*, 2013). Notably, upon amino acid deprivation the FLCN-FNIP complex inhibits the Rags via binding and stabilizing RagA/B in their inactive conformation (Lawrence *et al.*, 2019). Amino acid replenishment destabilizes the FLCN-FNIP-Rags complex through the lysosomal solute carrier family 38 member 9 (SLC38A9) (Rebsamen *et al.*, 2015). SLC38A9 is an Arginine-dependent amino acid transporter that binds RagA/B through its N-terminal tail, triggering FLCN dissociation from Rags and stimulating its GAP activity (Fromm, Lawrence and Hurley, 2020). SLC38A9 was also proposed acting as a GEF for RagA (Shen and Sabatini, 2018).

Moreover, leucine and glutamine are sensed by LARS (leucyl-tRNA synthetase) and ARF1 (ADP ribosylation factor 1), respectively, two activators of the mTORC1

pathway (Han *et al.*, 2012; Jewell *et al.*, 2015). Intriguingly, ARF1 functions through an unconventional Ragulator/Rags-independent mechanism (Jewell *et al.*, 2015; Meng *et al.*, 2020).

Finally, amino acids levels in lysosomes can be sensed by the V-ATPase (vacuolar ATPase), which interacts with Ragulator and modulates Rag GTPases activation (Zoncu *et al.*, 2011). The V-ATPase-Ragulator complex responds also to glucose starvation by activating AMPK and simultaneously inhibiting mTORC1 (Zhang *et al.*, 2014).



from Liu and Sabatini, Nat Rev Mol Cell Biol, 2020

Figure 4. Upstream regulators of the mTORC1 signaling pathway. Schematic model of the main pathways involved in lysosomal recruitment and activation of mTORC1.

Besides amino acids and glucose, also cholesterol has been shown to modulate mTORC1 via the SLC38A9-NPC1 (Niemann-Pick1) axis. NPC1 coordinates the

cholesterol export from the lysosome and, in cholesterol-depleted cells, it binds SLC38A9 and prevents its activity towards Rags (Castellano *et al.*, 2017).

2.3. Cellular pathways downstream of mTORC1

The mTORC1 signaling pathway controls the switch between catabolism and anabolism: in nutrient-rich conditions, mTORC1 activation triggers protein, nucleotide and lipid synthesis, whereas it inhibits degradative processes such as autophagy.

mTORC1 induces protein synthesis primarily through the phosphorylation of S6K1 (p70S6 kinase 1) and 4EBP1 (eukaryotic translation initiation factor 4E-binding protein 1). S6K and 4EBP1 contain a TOR signaling (TOS) motif, a five amino acid sequence, that is recognized by Raptor thereby allowing substrates recruitment by mTORC1 (Schalm and Blenis, 2002; Schalm *et al.*, 2003). The TOS motif enables specific metabolic responses via selective recruitment of mTORC1 substrates, indeed MiT-TFE factors lack a TOS motif and their phosphorylation is differentially regulated by mTORC1, as discussed below (Napolitano *et al.*, 2020).

4EBP1 is a protein translation inhibitor that prevents the assembly of the eIF4F complex through the sequestration of eIF4E. Multiple phosphorylation sites on 4EBP1 targeted by mTORC1 lead to its separation from eIF4E, consequently facilitating the initiation of cap-dependent translation (Hara *et al.*, 1997; Gingras *et al.*, 1999). S6K phosphorylation by mTORC1 activates its kinase activity leading to an increase in protein synthesis and cell proliferation. Indeed several proteins involved in mRNA translational initiation are substrates of S6K, including the ribosomal protein S6 and eIF4B (Holz *et al.*, 2005). S6K also enhances the activity of RNA polymerase I and III through phosphorylation of regulatory factors like UBF (upstream binding factor), TIF-1A (transcription initiation factor 1A) and MAF1 (Hannan *et al.*, 2003; Mayer *et al.*, 2004; Shor *et al.*, 2010).

Furthermore, mTORC1 stimulates lipid synthesis and inhibits lipogenesis, thus supporting the biogenesis of new membranes during cellular growth. mTORC1

induces the expression of key adipogenic components, such as PPAR-γ (peroxisome proliferator–activated receptor γ) and C/EBP-α (CCAAT/enhancer-binding protein α), probably in a 4EBP1-dependent mechanism (Le Bacquer *et al.*, 2007; Laplante and Sabatini, 2009). In addition, mTORC1 promotes *de novo* lipid synthesis in response to growth factors by activating SREBP-1 (Sterol regulatory element-binding protein 1), a transcription factor that regulates many lipogenic genes, including ACC (acetyl-CoA carboxylase), FASN (fatty acid synthase) and SCD-1 (stearoyl-CoA desaturase-1) (Porstmann *et al.*, 2008; Laplante and Sabatini, 2009). Besides, in adipocytes stimulated with insulin or amino acids, the phosphatidic acid phosphatase Lipin1 undergoes phosphorylation in a rapamycin-sensitive fashion, thus implying that mTOR signaling could directly influence Lipin1 activity (Huffman, Mothe-Satney and Lawrence, 2002). Lipin1 triggers lipogenesis through different mechanisms, including regulation of C/EBP-α4 and PPAR-γ expression and function (Phan, Péterfy and Reue, 2004; Koh *et al.*, 2008). Anyway, the effect of Lipin1 phosphorylation on lipid synthesis and gene expression is still debated.

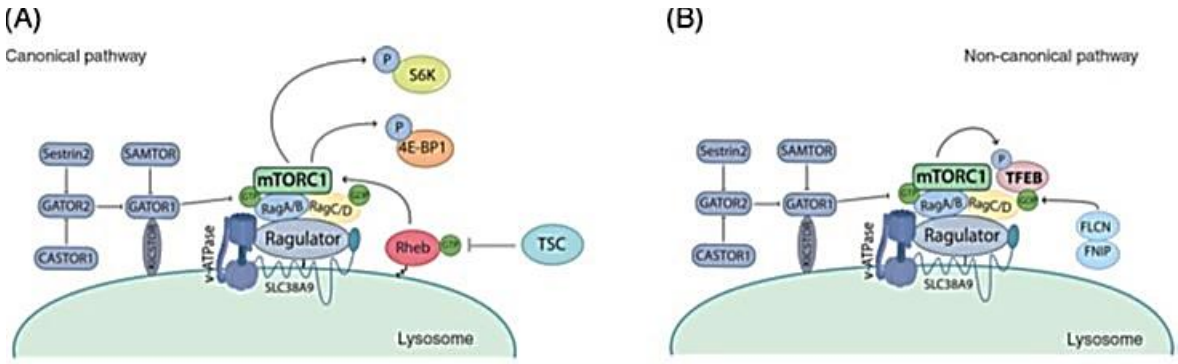
In addition, mTORC1 modulates also nucleotide synthesis by promoting *de novo* synthesis of purines and pyrimidines. mTORC1 stimulates purines production by controlling ATF4-dependent expression of MTHFD2 (methylenetetrahydrofolate dehydrogenase 2), a key enzyme of the mitochondrial tetrahydrofolate cycle (Ben-Sahra *et al.*, 2016). mTORC1 likewise induces the S6K-dependent phosphorylation of CAD, an enzyme responsible for the first three steps of pyrimidine biosynthesis (Ben-Sahra *et al.*, 2013).

In addition to triggering anabolic processes, mTORC1 suppresses catabolic programs. In particular, mTORC1 inhibits the autophagy, a self-degradative process of the cell that eliminates and recycles unnecessary or dysfunctional components. When nutrients are available, mTORC1 phosphorylates and suppresses the activity of crucial initiators of autophagy: ULK1 (unc-51-like autophagy-activating kinase 1) and several autophagy-related proteins. These proteins, together with FIP200 and

ATG101, orchestrate the process of autophagosome formation (Hosokawa *et al.*, 2009; Dikic and Elazar, 2018).

Moreover, mTORC1 is a negative regulator of UVRAG (UV radiation resistance-associated gene product), a protein that drives autophagosome and endosome maturation via binding to the HOPS (homotypic fusion and vacuole protein sorting) complex (Kim *et al.*, 2015).

Importantly, mTORC1 suppresses autophagy via the downregulation of TFEB, TFE3 and MITF, which are master transcriptional controllers of autophagy and lysosomal function (Napolitano and Ballabio, 2016). Intriguingly, mTOR-dependent phosphorylation of TFEB occurs through the FLCN–RagC/D axis, but it is insensitive to TSC-Rheb axis (Napolitano *et al.*, 2020). This fork of the mTORC1 pathway allows the differential regulation of its substrates, that can be identified as TOS-containing substrates (e.g. S6K, 4EBP1) and Rag-binding substrates (e.g. TFEB, TFE3) (Fig.5). Indeed, MiT-TFE factors lack a TOS motif, but they contain a Rag-binding region (RBR) in their N-terminus that allows them to interact with the Rags. Thereby, Rag GTPases directly bind TFEB, mediating its presentation to mTORC1. TFEB activity depends greatly on FLCN-Rags axis, in fact FLCN depletion is associated to TFEB constitutive activation (Petit, Roczniak-Ferguson and Ferguson, 2013).



from Napolitano, Di Malta and Ballabio, Trends Cell Biol, 2022

Figure 5. Canonical and non-canonical mTORC1 signaling pathways. Phosphorylation of TOS-containing substrates requires TSC-Rheb-dependent mTORC1 activation (A, canonical pathway), whereas phosphorylation of Rag-binding substrate (e.g. TFEB) occurs through the FLCN–RagC/D axis (B, non-canonical pathway).

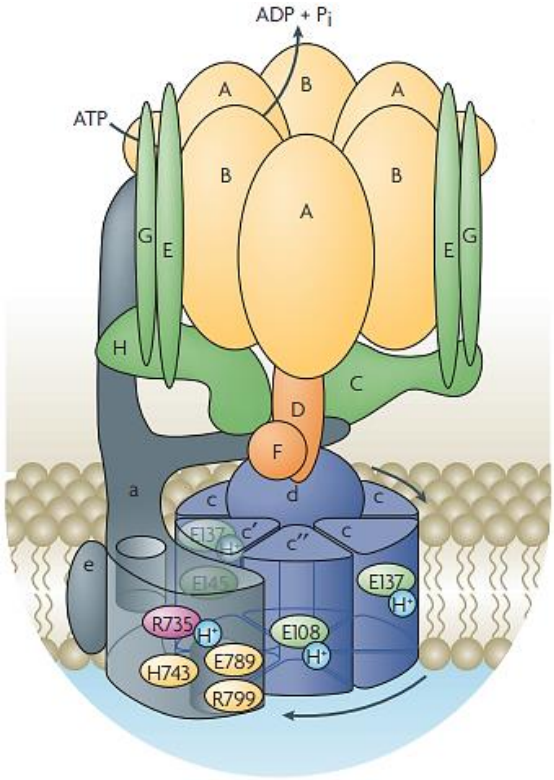
3. The vacuolar ATPase (V-ATPase)

The vacuolar (H^+) ATPase (V-ATPase) is an ATP-hydrolysis driven proton pump that is present at the plasma membrane or in the membrane of intracellular compartments, including lysosomes, vesicles, endosomes, and the Golgi apparatus (Stevens and Forgac, 1997). The V-ATPase couples the energy derived from ATP hydrolysis to the transport of protons across the membranes, through the mechanical rotation of its subunits (a mechanism known as rotational catalysis) (Futai *et al.*, 2019). By modulating proton (H^+) transport, the V-ATPase ensures the intraluminal low pH essential for various cellular processes, including membrane traffic, protein degradation, pH homeostasis, viruses and toxins entry, bone resorption by osteoclasts, and invasion of tumor cells.

3.1. Structure, function and regulation

The V-ATPase is a multiprotein complex consisting of at least 14 subunits that are organized in two large functional domains: the transmembrane V0 sector is composed by six subunits forming a proton channel (a, d, e, c, c', c'') whereas the cytosolic V1 sector contains eight different subunits (A-H) and is responsible for ATP hydrolysis (Stevens and Forgac, 1997; Kissing *et al.*, 2015). The catalytic region in the V1 sector is composed by three copies each of the A and B subunits, organized in alternating position. The EG heterodimers connected to subunits C or H form two peripheral stalks that serve as stators preventing rotation of the AB hexamer during ATP hydrolysis. The central stalk of the V1 domain contains subunits D and F and serves as a rotor connected to the V0 sector that rotates upon ATP hydrolysis (Cotter *et al.*, 2015). In particular, V1D and V1F subunits interact with V0d subunit which is positioned on the top of the proteolipid ring of the V0 domain, thereby energy from ATP hydrolysis is coupled to the proton transport. The proteolipid ring (or c ring) is composed of the hydrophobic subunits c, c' and c'' that contain a glutamic acid residue that can be protonated (Glu137, 145 and 108, respectively).

Protons are released from glutamic acid residues of the c ring to an arginine located in subunit a (Arg735). Subunit a has eight transmembrane helices forming the hemichannel which carries out proton transport across cellular membranes. Finally, the membrane-embedded subunit e has unknown function but is thought to anchor the V0 domain within the membrane, contributing to the overall structural integrity of the V-ATPase complex (Stevens and Forgac, 1997; Cotter *et al.*, 2015). Moreover, the V-ATPase interacts with two accessory proteins, ATP6AP1/AC45 and ATP6AP2/(pro)renin receptor (ATPase H^+ transporting accessory protein 1 and 2), that stabilizes the complex (Jansen and Martens, 2012; Kissing *et al.*, 2017).



Adapted from Forgac, Nat Rev Mol Cell Bio, 2007

Figure 6. V-ATPase structure and mechanism of action. The V-ATPase protein complex is formed by fourteen subunits organized in the cytoplasmic V1 domain and the membrane-embedded V0 sector. Energy from ATP hydrolysis is transmitted from the AB hexamer to the c-ring (c, c', c''), through the central stalk (D, F, d), thus leading rotation of the ring. Each protein of the proteolipid ring has a protonated glutamic acid residue (respectively E137, E145, E108) that, thanks to the rotatory mechanism, get in touch with and protonate an arginine residue in

subunit a (R735). Subunit a forms a hemichannel across the membrane that allows proton translocation.

The V-ATPase subunits exist in different isoforms that are tissue specific or localized in specific organelles. For instance, kidney and epididymis highly express the isoforms B1, C2b, E1, G3 of the V1 subunits, a4 and d2 isoforms in the V0 sector. The B1 isoform is also present in hair cells of the inner ear. The C2a isoform is specific of lungs. The E1 isoform is present in testis, olfactory epithelium and the acrosome. Synaptic vesicle acidification relies on expression of the G2 isoform in the central nervous system. Eventually, mammalian cells contain four isoforms of subunit a in the V0 domain: a1 is expressed in coated vesicles, a2 in early endosomes and Golgi apparatus, while a3 is found in late endosomes and lysosomes; a4 is specific of kidney and epididymis, as mentioned before. Furthermore, osteoclasts specifically express d2 and a3 isoforms at their plasma membrane (Toei, Saum and Forgac, 2010; Futai *et al.*, 2019).

V-ATPase activity is strictly controlled by different mechanisms, such as reversible assembly, trafficking to membranes, modulation of coupling efficiency and transcriptional regulation.

V1 and V0 assembly is reversible and rapid, thus allowing fast and specific response to different stimuli. Glucose starvation increases V-ATPase assembly in a mechanism dependent on AMPK and PI3K/Akt pathway (McGuire and Forgac, 2018). Amino acid withdrawal also promotes V-ATPase lysosomal assembly, a crucial step for amino acid-dependent inactivation of the mTORC1 complex (Stransky and Forgac, 2015). Furthermore, V-ATPase regulated assembly on lysosomes is particularly relevant for dendritic cells maturation, ensuring the acid pH required for antigen processing in response to LPS (lipopolysaccharide) (Trombetta *et al.*, 2003). This mechanism is dependent on both PI3K and mTOR pathways, since drugs targeting these kinases inhibit the increased assembly observed upon dendritic cells activation (Liberman *et al.*, 2014). PI3K, together with ERK (extracellular signal-

regulated kinase), has also been shown to induce V-ATPase assembly following infection of cells with influenza virus (Marjuki *et al.*, 2011). Finally, EGF (epidermal growth factor) also stimulates V-ATPase assembly, probably in order to promote lysosomes acidification and subsequent protein degradation (Xu *et al.*, 2012).

Another route of regulation of V-ATPase activity is through regulated trafficking. This mechanism is particularly important in polarized cells expressing the V-ATPase in their apical membrane, such as in kidney and epididymis. In these cells, a bicarbonate-sensitive adenylate cyclase increases cAMP levels in response to decrease in the cytoplasmic pH, leading to activation of PKA (protein kinase A) which directly phosphorylates the V1A subunits thus resulting in the apical membrane translocation of the pump (Cotter *et al.*, 2015; McGuire *et al.*, 2017). Additionally, V-ATPase trafficking at the plasma membrane is crucial for bone resorption led by osteoclasts that specifically express the a3 isoform on the ruffled border adjacent to bone. Intriguingly in osteoclast precursor cells a3 is localized to late endosomes and lysosome, and only upon differentiation a3-containing V-ATPase translocates to the plasma membrane, thus reflecting the importance of isoform expression and subcellular localization of the pump in different cellular context (Toyomura *et al.*, 2003).

Other stimuli can modulate V-ATPase activity without affecting its assembly or localization. Intriguingly, the transcription factor STAT3 can bind the V-ATPase and enhance its activity at the lysosome (Liu *et al.*, 2018). Moreover, some bacteria, like *Legionella Pneumophila* and *Salmonella Typhimurium*, block xenophagy after cell infection by secreting effectors that inhibits the V-ATPase (Xu *et al.*, 2010, 2019).

Modulation of the efficiency of coupling ATP hydrolysis with proton transport has been also proposed to regulate V-ATPase function. For instance, different isoforms of subunit a show dissimilar coupling efficiency in yeast, because of variations in their C-terminal domain (Kawasaki-Nishi, Nishi and Forgac, 2001). This feature would explain why the Golgi have a more alkaline pH than lysosomes.

Finally, V-ATPase transcriptional expression is under control of MiT/TFE factors, accordingly to the main role of these transcription factors in promoting autophagy and lysosomal biogenesis. The V-ATPase and TFEB participate in a feedback loop by which TFEB, which is a mTORC1 substrate, may in turn influence mTORC1 activity by controlling the expression of different subunits of the V-ATPase (Peña-Llopis *et al.*, 2011).

3.2. Physiological and pathophysiological roles of the V-ATPase

Lysosome homeostasis relies on the correct functioning of the V-ATPase that, by pumping H^+ ions inside the lysosomal lumen, guarantees the low pH necessary for a proper catalytic activity of degradative enzymes and hence degradation of macromolecules (Mindell, 2012). Unneeded cellular materials are delivered to lysosomes via endocytosis, chaperone-mediated autophagy, or macroautophagy, thereby V-ATPase activity is essential to ensure breakdown of luminal contents. In particular, during autophagy, unnecessary macromolecules and organelles are engulfed within autophagosomes, which are double-membraned vesicles that fuse with lysosome to form autolysosomes (Glick, Barth and Macleod, 2010). During this process, acidification is crucial for autophagosome/lysosome fusion and for degradation of the luminal contents; indeed V-ATPase inhibitors, such as Bafilomycin A1 and Concanamycin A, block the autophagic flux and are also referred as autophagy inhibitors.

Endosomal acidification plays a fundamental role in the recycling of internalized receptors derived from receptor-mediated endocytosis, since the low pH allows the release of the ligands, enabling the return of receptors back to plasma membrane (Maxfield and McGraw, 2004). Furthermore, V-ATPase-dependent endosomal acidification modulates endocytosis by driving the recruitment of the small GTPase Arf6 and its GEF ARNO (ARF-nucleotide-binding-site opener), two endocytosis effectors implicated in carrier vesicle-coat formation and actin cytoskeletal remodeling (Hurtado-Lorenzo *et al.*, 2006). Additionally, the acidic pH in late

endosomes controls delivery and release of newly synthetized proteases in lysosome, while enabling the return of mannose 6-phosphate receptor to the trans-Golgi (Ghosh, Dahms and Kornfeld, 2003).

Notably, several pathogens take advantage of the endocytic pathway to entry into the cytoplasm and replicates. In fact, endosomal acidic pH allows fusion of enveloped viruses, like Influenza and Ebola, with the endosomal membrane and release of their viral genome into the cytosol (Grove and Marsh, 2011). The Anthrax toxin also exploits the acidic endosomes to reach the host cytoplasm (Gruenberg and van der Goot, 2006).

The V-ATPase modulates different vital cellular processes; thus, it is not surprising that its activity is altered in different diseases. First, V-ATPase promotes cancer cell survival, growth and invasiveness. V-ATPase-dependent degradative pathways sustain tumor cells during hypoxia and nutrient withdrawal (Stransky, Cotter and Forgac, 2016). In addition, increased plasma membrane levels of the V-ATPase have been detected in several invasive tumors, including breast, lung, liver and pancreatic cancer (Cotter *et al.*, 2015). It has been proposed that V-ATPase overexpression in the plasma membrane is beneficial for cancer cells because it boosts acidification of the tumor microenvironment, and subsequently promotes both invasiveness of metastatic cells and drug resistance (Collins and Forgac, 2020).

Recent studies are pointing out the role of the V-ATPase in neurodegenerative disorders. Neurodegenerative diseases often show lysosomal alkalinization or dysfunction, resultant in accumulation of undigested materials. Mutations of ATP6AP2 have been associated with different forms of Parkinsonism, intellectual disability and epilepsy, probably because of defective autophagy (Rujano *et al.*, 2017; Song *et al.*, 2020). Additionally, in neurons the V-ATPase bind LRRK2 (leucine-rich repeat kinase 2), a protein mutated in most cases of familiar Parkinson disease (Wallings, Connor-Robson and Wade-Martins, 2019). A common mutation of LRRK2 (R1441C/G) was found to decrease the binding with the subunit a1 of the

V-ATPase, leading to increase lysosomal pH and reduction of autophagosome/lysosome fusion.

Several studies suggest that V-ATPase deficiency may be also linked to Alzheimer's disease, likely because loss of function of Preselin-1, a component of the γ-secretase involved in the processing of the amyloid precursor protein, causes decreased targeting of the V-ATPase to lysosomes (Lee *et al.*, 2015; Song *et al.*, 2020).

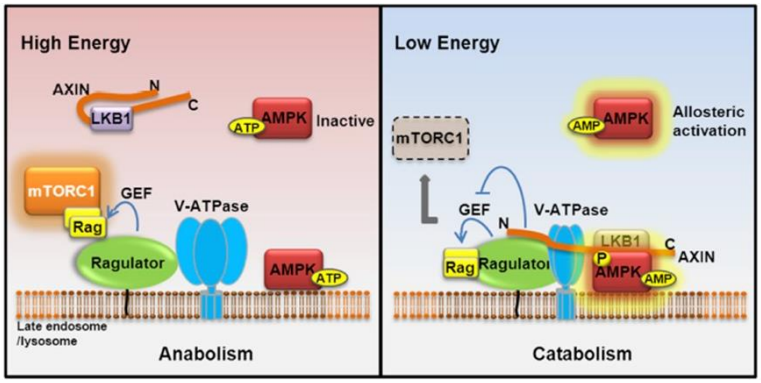
Finally, loss-of-function mutations in different V-ATPase subunits have been identified in a variety of human diseases. For instance, V0a2 was discovered mutated in the autosomal recessive cutis laxa type II, V0a3 in a severe form of infantile osteoporosis, while V0a4 and V1B1 mutations causes recessive distal renal tubular acidosis with deafness (Collins and Forgac, 2020; Song *et al.*, 2020).

3.3. The V-ATPase in nutrient sensing: the mTORC1 and AMPK pathways

Emerging evidence suggests the V-ATPase is involved in the regulation of mTORC1 activity by interacting with lysosomal complex Ragulator, thus participating in the lysosomal amino acid sensing machinery, even though its role is not completely understood (Zoncu *et al.*, 2011; Bar-Peled and Sabatini, 2014). The binding of the V-ATPase with Ragulator and Rag GTPases is amino acid-dependent. The V-ATPase-Ragulator interaction is weakened upon amino acid replenishment, but interestingly the V-ATPase inhibitor Salicylihalamide A prevents amino acids from impairing this binding (Zoncu *et al.*, 2011). Notably, V-ATPase inhibition downregulates the mTORC1 pathway impairing mTOR lysosomal localization. Intriguingly, the V-ATPase/autophagy activator EN6 also inhibits mTORC1 activity and lysosomal recruitment (Chung *et al.*, 2019). This compound uncouples the V-ATPase from the Ragulator-Rags complex, leading to mTORC1 inactivation. Together these studies underline the essential role of the V-ATPase-Ragulator-Rags complex in the modulation of the mTORC1 signaling.

The V-ATPase-Ragulator complex is also required for the activation of AMPK, serving as a switch between catabolism and anabolism (Zhang *et al.*, 2014). Glucose

starvation promotes V-ATPase lysosomal assembly, which is required for AMPK activation. In fact, the V-ATPase recruits Axin, a scaffold protein that mediates the binding of AMPK and its kinase LKB1 (liver kinase B1), thereby triggering the AMPK signaling pathway (Zhang *et al.*, 2013, 2014).



from Zhang et al., Cell Metab, 2014

Figure 7. V-ATPase-Ragulator complex senses the switch between mTOR and AMPK activation. In presence of nutrients, the V-ATPase weakly interact with Ragulator and mTORC1 is active. Upon glucose starvation, the strong V-ATPase-Ragulator complex is bound by Axin that stimulates recruitment and activation of AMPK, and simultaneously mTORC1 is inhibited.

4. The Birth-Hogg-Dubé syndrome

The Birt-Hogg-Dubé (BHD) syndrome is rare genetic disorder that primarily affects skin, lungs, and kidneys. BHD prevalence and incidence are unclear because the disease is difficult to diagnose, anyway its prevalence is estimated at about 1/200'000 [www.orpha.net]. The BHD is an autosomal dominant disease caused by mutations in the *FLCN* gene (folliculin), which is located on the short arm of chromosome 17 (Khoo *et al.*, 2001; Nickerson *et al.*, 2002).

4.1. BHD clinical features

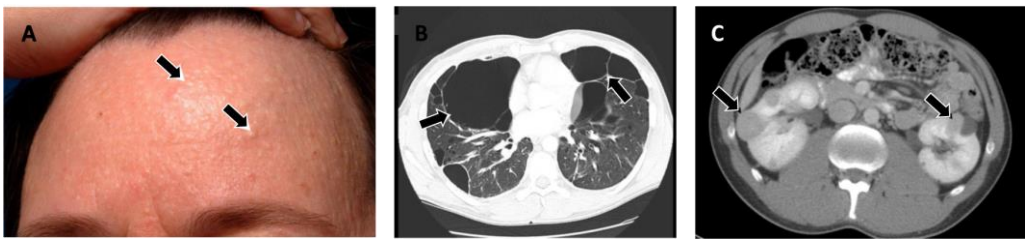
The BHD is characterized by the following key features (Fig.8), even though the symptoms can vary in occurrence and severity:

- skin manifestations (Fig.8a): trichodiscomas, acrochordons and fibrofolliculomas are benign skin tumors that typically appear on face, neck, and upper chest. These

skin lesions are usually small, skin-colored or whitish, and they are the most common manifestation of the syndrome, as well as the first to appear (Schmidt and Linehan, 2015; Tellechea *et al.*, 2015)

- lung abnormalities (Fig.8b): lung cysts can cause spontaneous pneumothorax, that is the collapse of lungs due to the rupture of these fluid-filled sacs (Schmidt e Linehan 2015; Marciniak e Johnson 2020)

- kidney cysts and tumors (Fig.8c): BHD patients have increased risk of developing kidney tumors (7-fold greater than unaffected people). BHD-associated kidney tumors may differ in histology and aggressiveness, but they can be classified into four main types: chromophobe renal cell carcinoma, clear cell carcinoma, papillary carcinoma and renal oncocytoma (Schmidt and Linehan, 2015; Hasumi *et al.*, 2016).



Adapted from Schmidt and Linehan, Nat Rev Urol, 2015

Figure 8. BHD pathological manifestations. Multiple fibrofolliculomas (A), bilateral multiple pulmonary cysts (B) and bilateral multifocal tumors (C) are the main symptoms of the Birt-Hogg-Dubé.

The age of onset of BHD can vary among affected individuals, even within the same family, but in most cases first clinical manifestations appear during the third or fourth decade of life (Schmidt and Linehan, 2015). Dermatological manifestations are often predictive of kidney tumors.

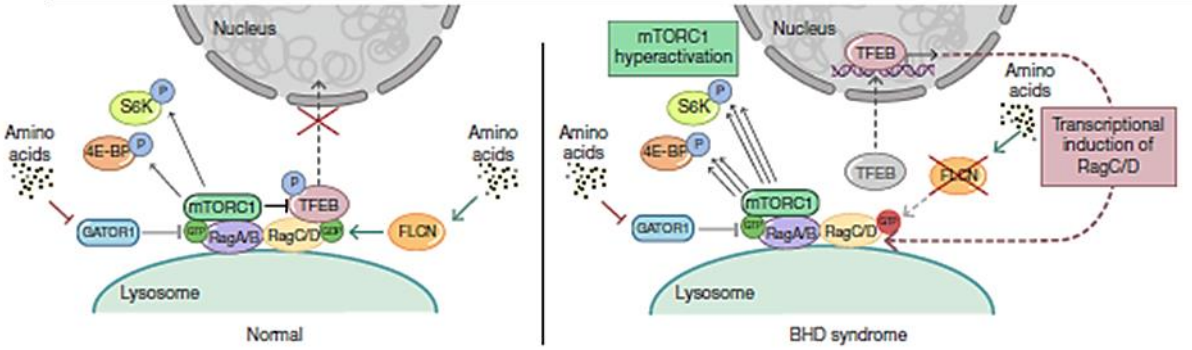
Because of its rarity and its clinical variability, BHD is likely underdiagnosed and mistaken with other pulmonary or renal diseases. Its diagnosis relies on a combination of clinical evaluation and genetic testing to identify mutations in the *FLCN* gene (Daccord *et al.*, 2020). There is no specific treatment for the BHD

syndrome, therefore early diagnosis, prevention and management of the symptoms are main therapeutic approaches. Currently, excision and surgery are the only therapies available for fibrofolliculomas and renal cancers associated to the BHD syndrome (Schmidt and Linehan, 2015; Daccord *et al.*, 2020).

4.2. BHD molecular genetics: the role of FLCN, mTOR and MiT/TFE factors

The BHD is referred as a hereditary cancer syndrome, because germline mutations in *FLCN* gene predispose the affected individuals to the development of tumors. FLCN is a tumor suppressor that fits Knudson two-hit model: inactivation of the normal FLCN allele by somatic mutation or chromosomal loss results in loss of heterozygosity (LOH) and tumor initiation (Vocke *et al.*, 2005; Schmidt and Linehan, 2015, 2018). Different *in vivo* models supported this mechanism of pathogenesis in BHD-associated tumors, mostly in kidney manifestations (Hasumi *et al.*, 2009; Schmidt and Linehan, 2018).

As discussed above, FLCN regulates different anabolic and catabolic processes by modulating mTOR and AMPK pathways (Baba *et al.*, 2006, 2008; Hasumi *et al.*, 2009). Although FLCN is a positive regulator of mTOR, the BHD syndrome is associated with mTORC1 hyperactivation but impaired mTORC1-mediated phosphorylation of TFEB and TFE3 (Baba *et al.*, 2008; Wada *et al.*, 2016; Napolitano *et al.*, 2020). This paradox is explained by the mTORC1-TFEB/TFE3 feedback loop whereby TFEB and TFE3 activation promotes mTORC1 activity by transcriptionally regulating the levels of RagC and RagD GTPases (Fig.9) (Di Malta *et al.*, 2017; Napolitano *et al.*, 2020). MiT/TFE constitutive activation is the main driver of kidney cancer in the BHD syndrome, and we recently showed that both TFEB and TFE3 contribute to kidney cyst formation and hyperactivation of mTORC1 in kidney-specific Flcn-KO mice, albeit in distinct and cooperative ways (Napolitano *et al.*, 2020; Di Malta *et al.*, 2023).



From Napolitano, Di Malta et al., Nature, 2020

Figure 9. Molecular basis of BHD pathogenesis. In normal condition (left), the activation of Rag GTPases by amino acids elicits mTORC1-dependent phosphorylation of S6K, 4E-BP1 and TFEB, which is retained in the cytoplasm. In BHD syndrome (right), the absence of FLCN leads to the nuclear translocation of TFEB and, as a consequence, hyperactivation of mTORC1 via induction of RagC/D expression.

RESULTS

1. Silencing of the V-ATPase promotes TFEB cytosolic re-localization in a mTORC1-dependent manner

Our previous studies demonstrated that TFEB and TFE3 constitutive activation significantly contributes to the development BHD-associated kidney cysts and tumors (Napolitano *et al.*, 2020; Di Malta *et al.*, 2023). Therefore, these transcription factors represent appealing therapeutic targets for the treatment of the BHD syndrome. In order to identify correctors of TFEB cellular localization in BHD cellular models, we performed a High Content Screening (HCS) in FLCN-knockout HeLa cells exploiting a library of siRNAs targeting "druggable genes" (about 11,000 genes) and lysosomal membrane proteins (229 genes). Interestingly, the most significant hits identified by this screening were genes encoding subunits of vacuolar H^+-ATPase (V-ATPase), in particular *ATP6V0C* and *ATP6V1A* (Fig.10).

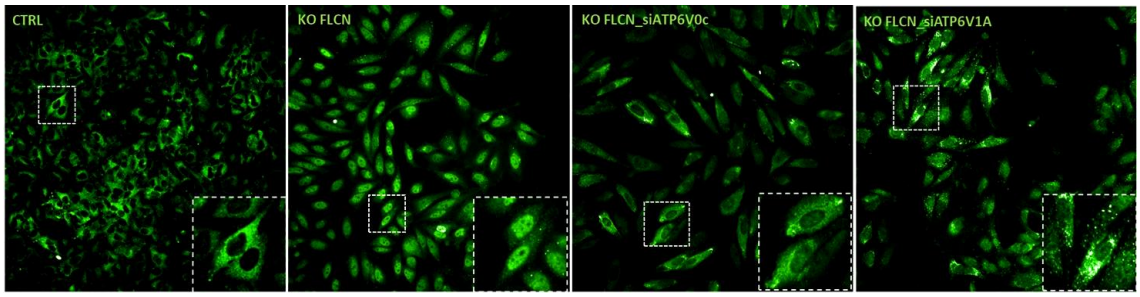


Figure 10. Silencing of V-ATPase subunits promotes TFEB nuclear export in FLCN-KO HeLa cells. Immunofluorescence analysis of endogenous TFEB from control HeLa (CTRL) or FLCN-KO HeLa transfected with the indicated siRNAs. Insets show magnification of selected area.

TFEB cytoplasmic retention is promoted by mTORC1-mediated phosphorylation in a RagC/D dependent fashion in presence of amino acids (Bar-Peled and Sabatini, 2014; Napolitano *et al.*, 2020). Therefore, we asked whether TFEB subcellular localization was sensitive to amino acid availability in FLCN-KO HeLa cells after ATP6V0C or ATP6V1A depletion. We found that silencing of these subunits promotes

TFEB cytosolic re-localization in a mTORC1-dependent manner, since TFEB localization remains sensitive to amino acid deprivation and treatment with Torin1 (an inhibitor of mTOR activity) (Fig.11a). Similar results were obtained in HK-2 FLCN KO cells stably expressing an exogenous TFEB-GFP in a doxycycline- inducible manner (Fig.11b).

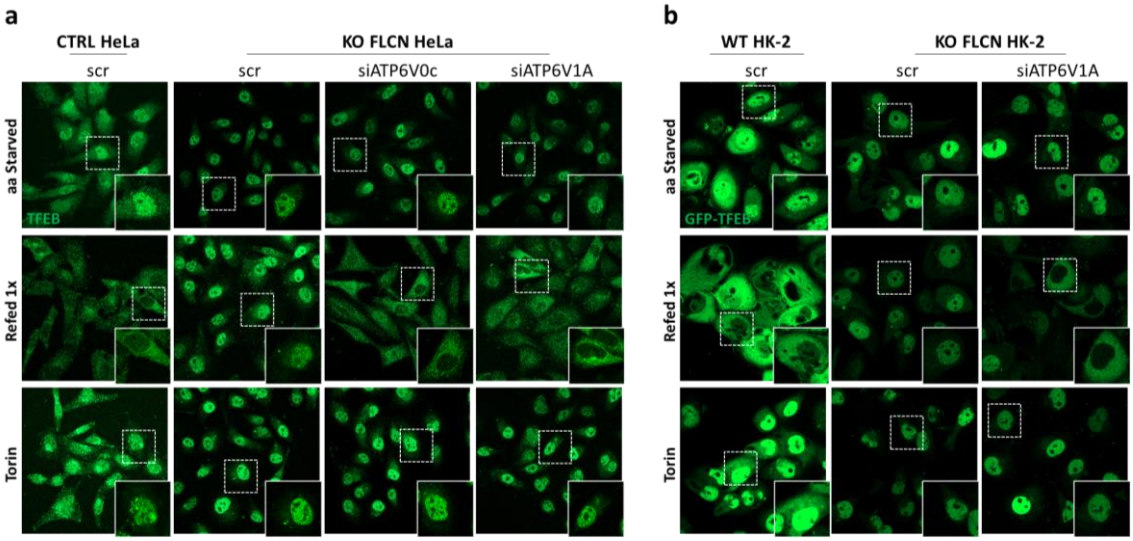


Figure 11. V-ATPase silencing promotes TFEB cytosolic re-localization in an amino-acid-sensitive manner. Immunofluorescence of endogenous TFEB in HeLa (**a**) or TFEB-GFP in HK-2 (**b**) FLCN-KO relative to the correspondent control cells, transfected for 72h with the indicated siRNAs (siATP6V0C or siATP6V1A) or scramble (scr) siRNA, and subjected to either amino acid starvation for 1h, or starvation and refeeding for 1h in the presence or absence of 1μM Torin. Cells in (**b**) were treated with 2,5μg/ml doxycycline for 24h.

We found that silencing of the V-ATPase was able to increase the cytosolic levels of TFEB protein in FLCN-KO cells (Fig.12), and we also observed that TFEB phosphorylation at mTORC1 target sites was increased, particularly for the serine 142 (Fig.13). Notably, phosphorylation of the mTORC1 substrates S6K, S6 and 4E-BP1was also increased upon V-ATPase silencing (Fig.13,14).

Together, these data suggest that, in absence of FLCN, depletion of the V-ATPase promotes TFEB phosphorylation and cytoplasmic localization in a mTORC1-dependent manner.

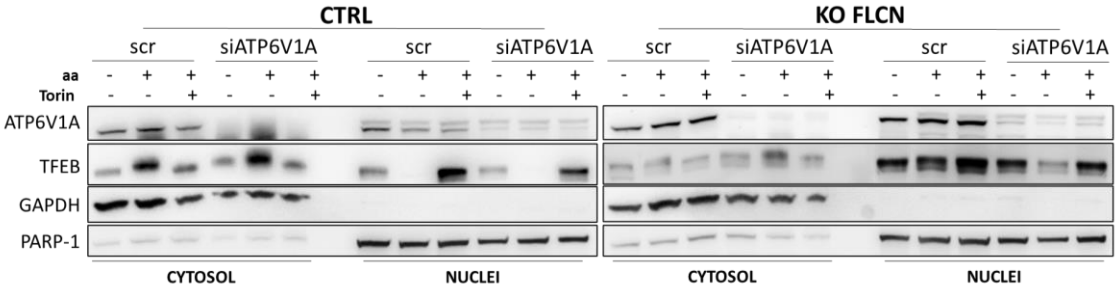
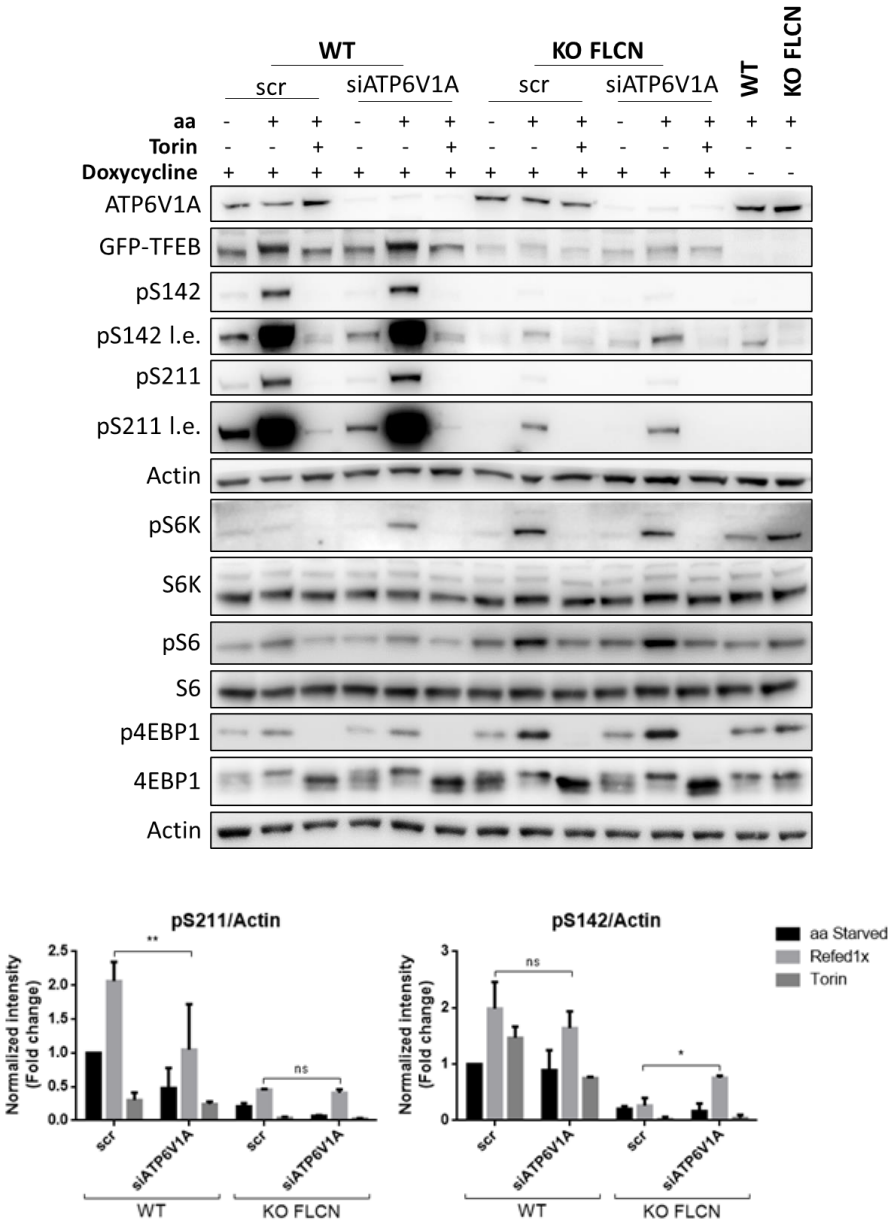


Figure 12. Silencing of the V-ATPase rescues TFEB localization in FLCN-KO cells. Western blot analysis of the indicated proteins in cytosolic and nuclear fractions from FLCN-KO HeLa silenced for the ATP6V1A subunit or for scramble. Cells were starved of amino acids for 1h, or starved and refed for 1h in presence or absence of 1μM Torin. GAPDH and PARP-1 were used as loading control for cytosolic and nuclear fractions, respectively.



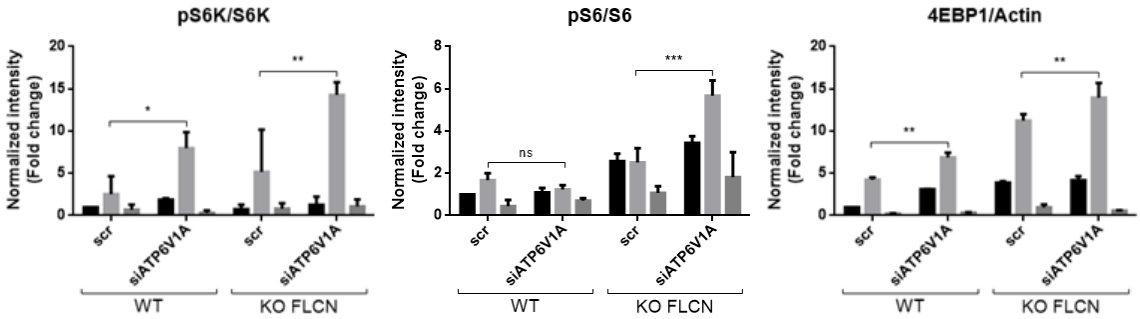


Figure 13. mTORC1 activity is promoted by V-ATPase silencing in FLCN-KO HK-2. Representative immunoblotting and quantification (mean±s.d.; n=3 experiments) of HK-2 cells transfected for 72h with siATP6V1A or control siRNA, and subjected to either amino acid starvation and refeeding for 1h in the presence or absence of 1μM Torin. Plots show pS142/actin, pS211/actin, pS6K/S6K, pS6/S6 and p4E-BP1/actin ratios. *p=0.01, **p=0.001, ***p<0.0001, ns= not significant. Tukey's multiple comparisons test. l.e.=long exposure.

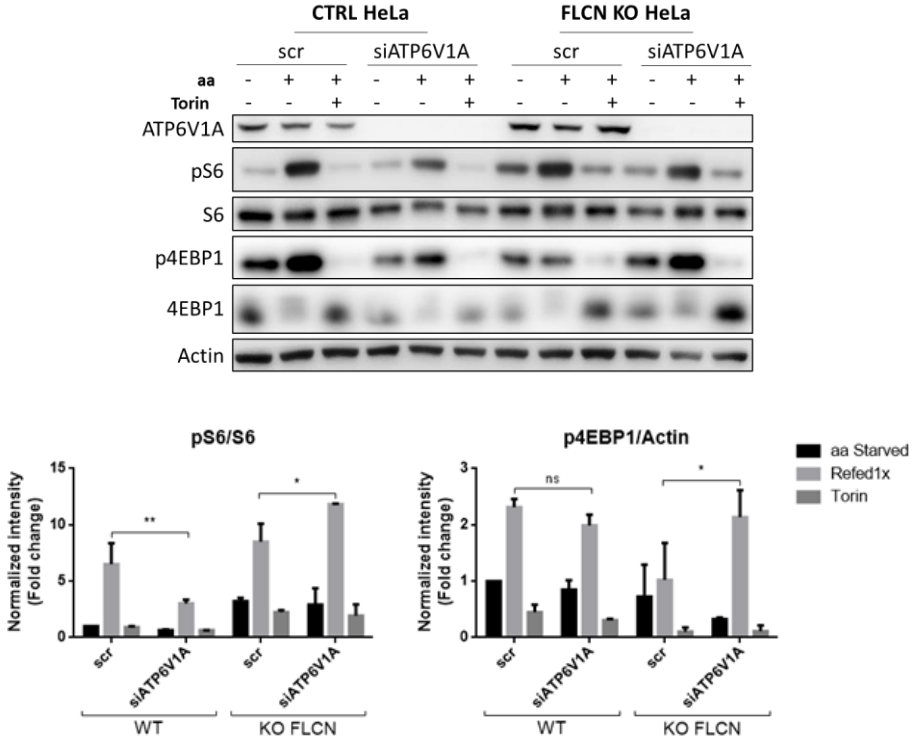


Figure 14. mTORC1 activity is promoted by V-ATPase silencing in FLCN-KO HeLa. Representative immunoblotting and quantification (mean±s.d.; n=3 experiments) of HeLa transfected for 72h with siATP6V1A or control siRNA, and subjected to either amino acid starvation and refeeding for 1h in the presence or absence of 1μM Torin. Plots show pS6/S6 and p4E-BP1/actin ratios. *p=0.01, **p=0.001, ns= not significant. Tukey's multiple comparisons test.

2. AMPK signaling is not affected by V-ATPase downregulation in FLCN-KO cells

The V-ATPase is important for activation of AMP-activated protein kinase (AMPK), another crucial energy sensor for cellular metabolic control (Zhang *et al.*, 2014). Under energy starvation conditions, AMPK negatively regulates mTORC1, primarily through phosphorylation and activation of TSC2 (Inoki, Zhu and Guan, 2003). Moreover, several studies identified FLCN and FNIP1/2 as AMPK binding partners, but no clear function is known for this interaction (Baba *et al.*, 2006; Paquette *et al.*, 2021; Malik *et al.*, 2023). The intricated interplay of these proteins leads us to investigate the AMPK pathway in FLCN-KO cells after downregulation of the V-ATPase. Notably, we could not detect any major alteration in the phosphorylation of both AMPK and the Acetyl-CoA carboxylase (ACC) - a substrate of AMPK - in FLCN-KO cell lines upon ATP6V1A silencing (Fig.15a,b).

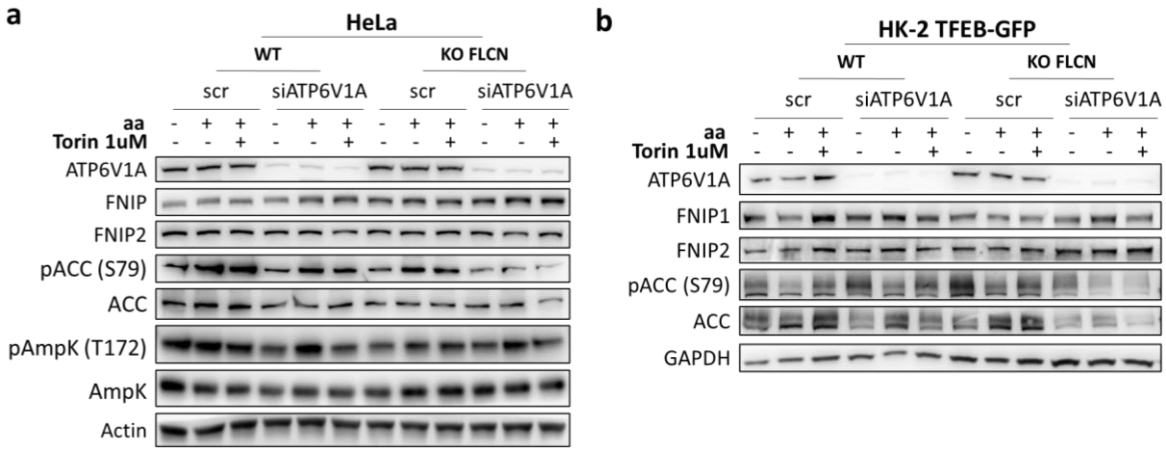


Figure 15. AMPK activity is not affected by V-ATPase silencing. Immunoblotting analysis of HeLa (**a**) and HK-2 (**b**) WT and FLCN-KO transfected for 72h with siATP6V1A or control siRNA, and subjected to either amino acid starvation and refeeding for 1h in the presence or absence of 1μM Torin.

3. Silencing of different V-ATPase subunits promotes TFEB cytoplasmic re-localization in FLCN-KO cells

The V-ATPase is a large multisubunit complex composed of fourteen subunits divided in two functional domains (V_1 and V_0) plus two accessory proteins (ATP6AP1 and ATP6AP2) (Cotter *et al.*, 2015; Kissing *et al.*, 2017). The V_0 sector consists of six different subunits that form the proton translocation domain. The V_1 sector is comprised of eight subunits and it represents the ATP-hydrolytic domain. ATP6AP1 and ATP6AP2 are key accessory proteins and important factor for V-ATPase assembly (Jansen and Martens, 2012).

Our HCS screening highlighted the V0c and the V1A subunits as top genes whose silencing promotes TFEB cytoplasmic re-localization in FLCN-KO cells. We tested siRNAs targeting other V-ATPase subunits, in particular the membrane embedded V0d1 and the cytoplasmic V1D, and we found that silencing of both subunits was associated with TFEB cytoplasmic re-localization in a mTOR-dependent manner in FLCN-KO cells (Fig.16a, 17a,b). However, the silencing of the accessory protein ATP6AP2 was not sufficient to rescue TFEB localization in FLCN-KO cells, thus suggesting that the structural integrity of the V-ATPase is important to mediate the regulation of TFEB in FLCN-KO cells (Fig.16b, 17c).

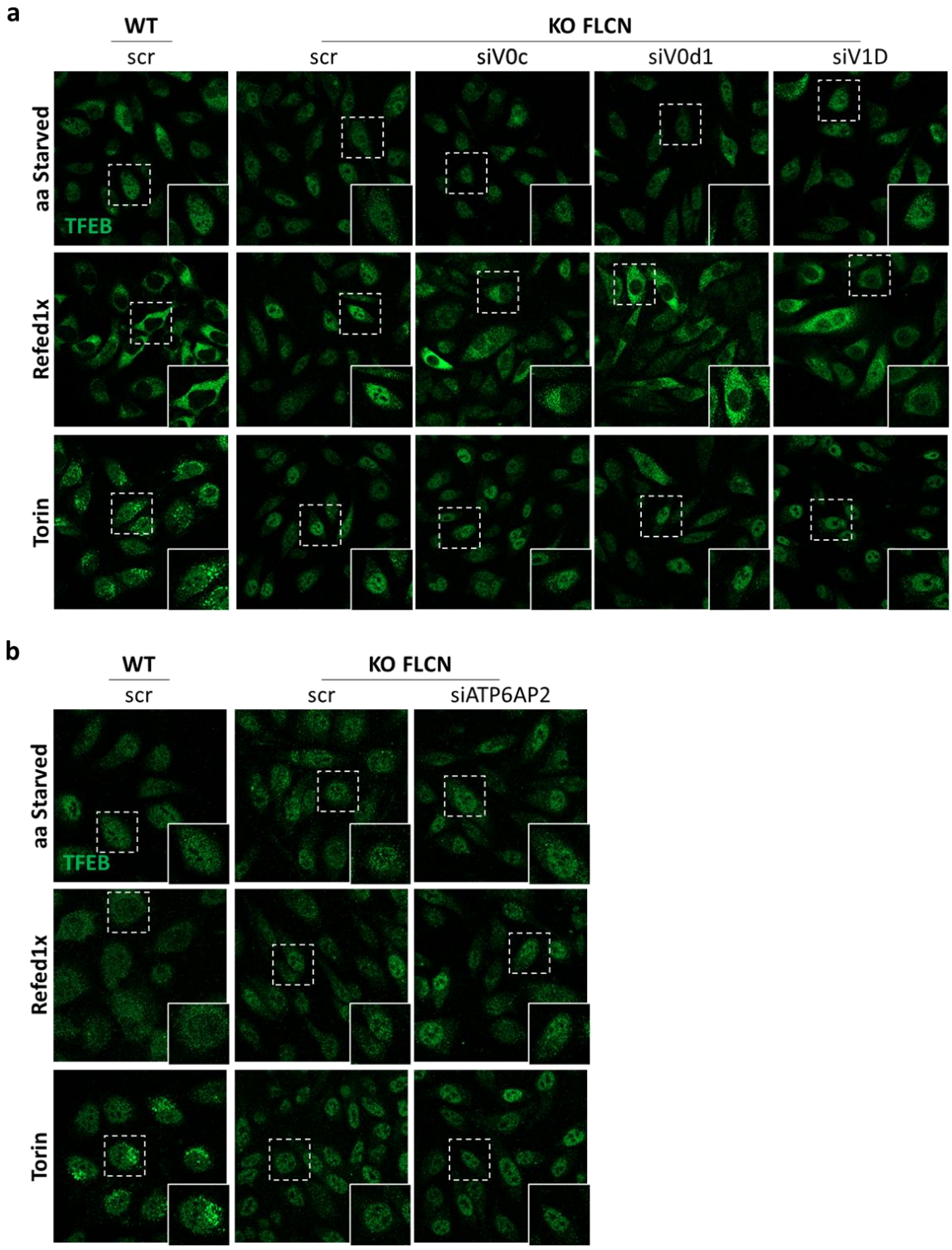


Figure 16. Silencing of different V-ATPase subunits differentially affects TFEB cellular localization. Immunofluorescence of endogenous TFEB localization in WT or FLCN-KO HeLa transfected for 72h with siRNAs targeting the following V-ATPase genes: *ATP6V0C*, *ATP6V0D1*, *ATP6V1D* (**a**) and *ATP6AP2* (**b**), upon amino acid starvation or refeeding for 1h, in presence or absence of 1μM Torin.

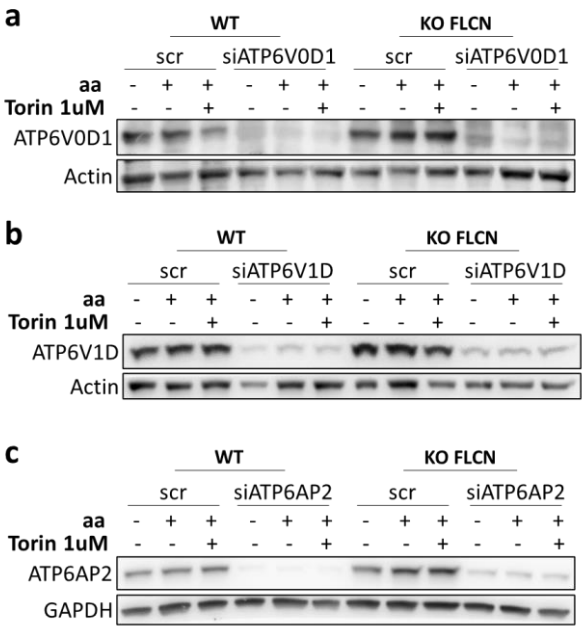


Figure 17. Validation of the silencing of different V-ATPase subunits. Immunoblot analysis of cells showed in Fig.15, silenced for *ATP6V0D1* (**a**), *ATP6V1D* (**b**) and *ATP6AP2* (**c**).

4. Silencing of the V-ATPase also rescues TFE3 cytosolic re-localization in FLCN-KO cells

Previous studies clearly demonstrated that TFE3 role largely overlaps with that of TFEB and that, similar to TFEB, TFE3 activity is strictly controlled by FLCN-mTORC1 axis, being constitutively active in FLCN-KO cellular and murine models (Puertollano *et al.*, 2018; Di Malta *et al.*, 2023). In order to test whether the silencing of the V-ATPase could promote TFE3 cytosolic re-localization in FLCN-KO cells, as observed for TFEB, we analyzed the localization of endogenous TFE3 in FLCN-KO HeLa and HK-2 cells in control condition (cells transfected with scramble siRNA) or upon silencing of ATP6V1A. As found for TFEB, our results clearly indicate that the silencing of the V-ATPase promotes TFE3 cytoplasmic re-localization in a mTORC1-dependent manner (Fig.18).

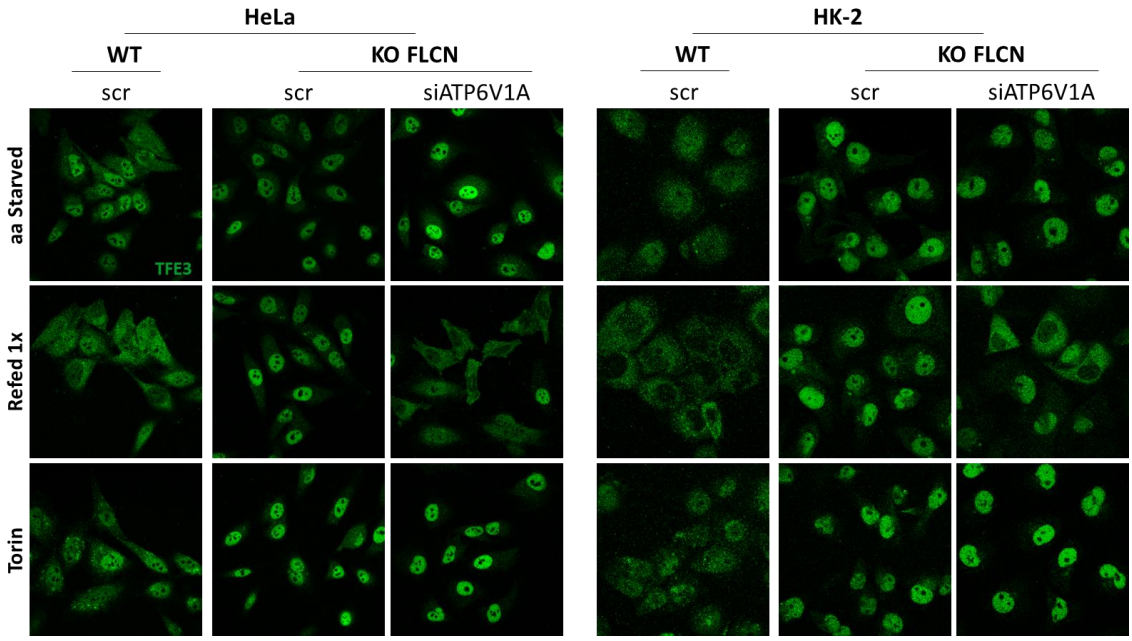


Figure 18. In FLCN-KO cells TFE3 re-localize to the cytosol upon silencing of the V-ATPase. Immunofluorescence analysis of endogenous TFE3 in FLCN-KO HeLa transfected with siATP6V1A or with control siRNA and subjected to either amino acid starvation for 1h, or starvation and refeeding for 1h in the presence or absence of 1μM Torin.

5. V-ATPase downregulation decreases gene expression of TFEB/TFE3 target genes

TFEB and TFE3 are members of the MiT/TFE family of transcription factors, key modulators of lysosome biogenesis and metabolism (Napolitano and Ballabio, 2016). These transcription factors bind the CLEAR regulatory motif in the promoter of genes involved in the mTORC1-signaling and the lysosomal–autophagic pathways (Settembre, Fraldi, *et al.*, 2013; Ballabio and Bonifacino, 2020). In absence of FLCN, TFEB/TFE3 constitutive activation leads to overexpression of its target genes and hyperactivation of these pathways (Di Malta *et al.*, 2023). We evaluated whether the silencing of the V-ATPase, by favoring TFEB/TFE3 cytosolic re-localization, may result in the downregulation of the expression levels of their target genes in FLCN-KO cells. In line with our hypothesis, we observed that silencing of the ATP6V1A subunit resulted in the downregulation of several TFEB/TFE3 target genes in FLCN-KO cells relative to cells transfected with scramble siRNA (Fig.19b). These results suggest that the downregulation of V-ATPase activity may represent a valuable approach to limit TFEB/TFE3 hyperactivation in the BHD syndrome.

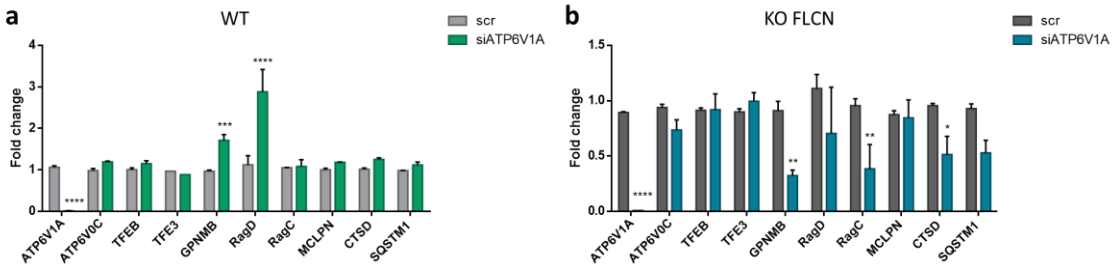


Figure 19. V-ATPase downregulation decreases the transcriptional levels of TFEB/TFE3 targets. mRNA levels of different TFEB/TFE3 target genes in WT (**a**) and FLCN-KO (**b**) HeLa cells silenced for V1A subunit of the V-ATPase. Values are normalized relative to HPRT1 and expressed as fold change relative to control samples (silenced for scramble). Results are mean±s.e.m.; n=3. *p=0.01, **p=0.001, ***p<0.0001. Sidak's multiple comparisons test.

6. Silencing of the V-ATPase promotes mTOR and RagC interaction with TFEB

In order to better understand the role of the mTORC1 pathway in this mechanism, we analyzed the lysosomal recruitment of mTOR and RagC. In response to amino acid availability, mTOR is recruited to the lysosome where Rag GTPases serve as a docking site (Sancak *et al.*, 2010). We found that colocalization of mTOR with the lysosomal marker LAMP1 increases in FLCN-KO cells after ATP6V1A depletion (Fig.20). Nonetheless, preliminary results showed that RagC lysosomal recruitment is not significantly altered in FLCN-KO cells silenced for ATP6V1A (Fig.21).

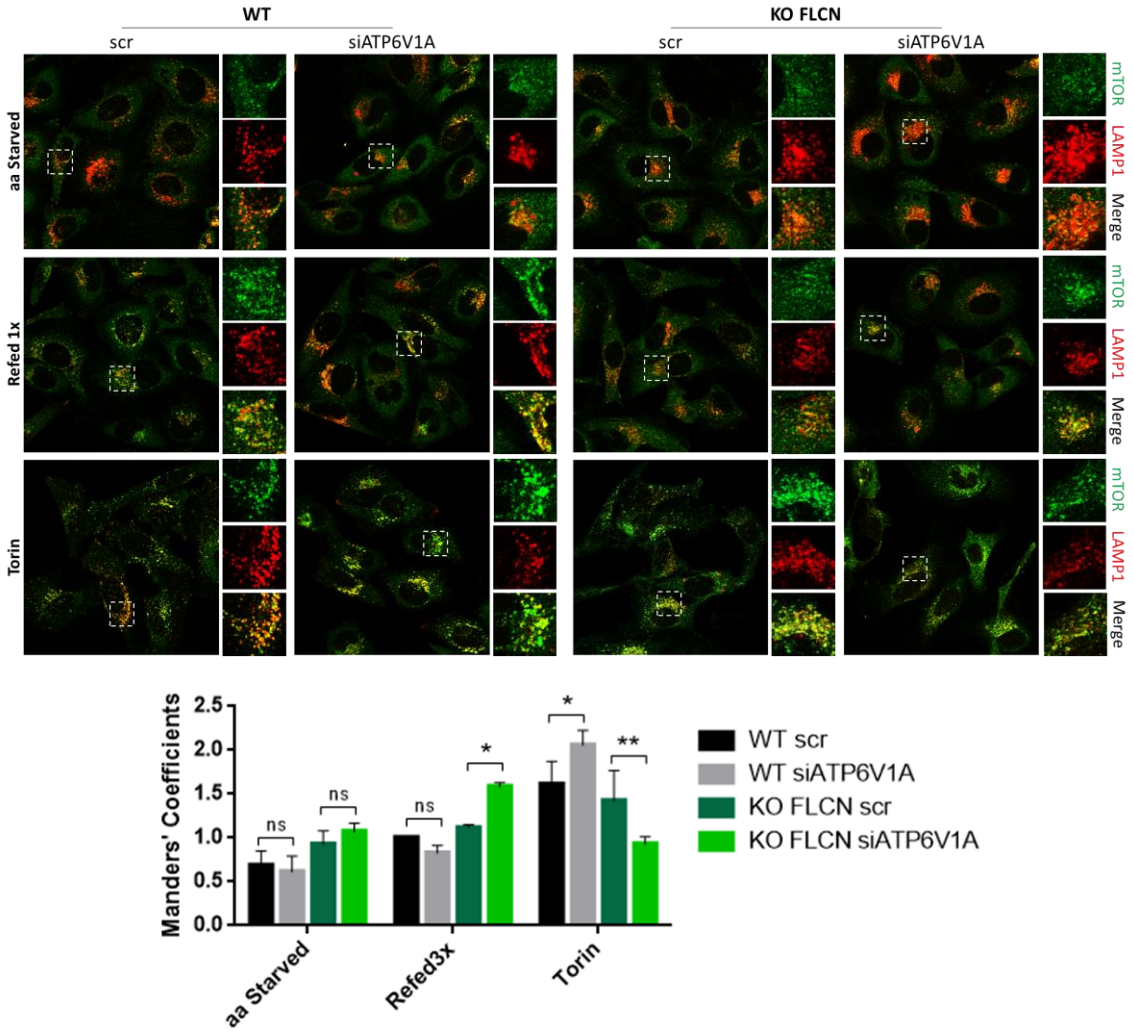


Figure 20. mTOR lysosomal recruitment increases upon V-ATPase silencing in FLCN-KO HeLa. Representative images of WT and FLCN-KO Hela transfected for 72h with siRNA targeting ATP6V1A or scramble, then stained with mTOR and Lamp1 antibodies. The graph below represents the colocalization analysis of mTOR and LAMP1. Colocalization results are expressed as Menders'

colocalization coefficient. Results are mean±s.d. n=3 independent experiments. *p=0.01, **p=0.001, ns= not significant. Tukey's multiple comparisons test.

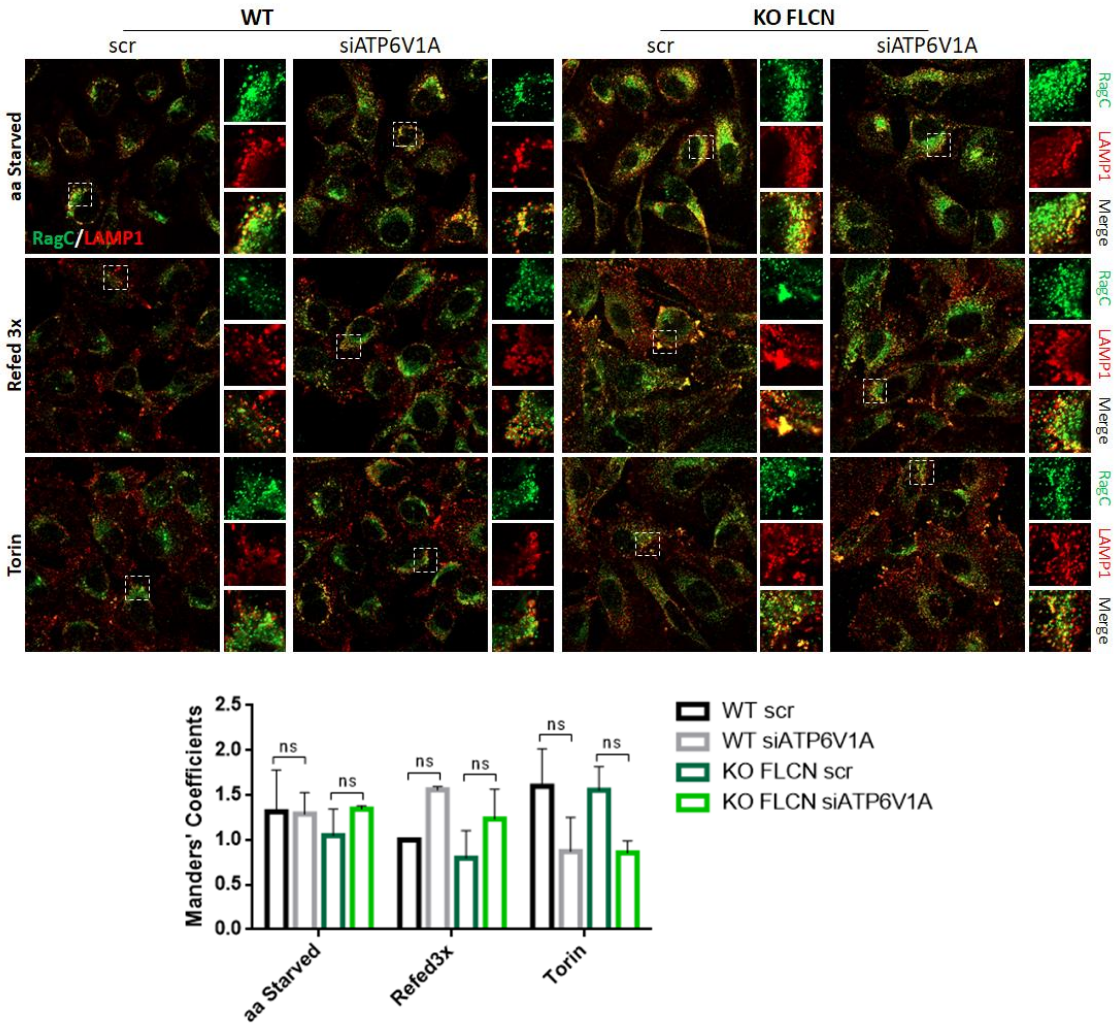


Figure 21. RagC lysosomal recruitment is not affected by V-ATPase silencing in FLCN-KO HeLa. WT and FLCN-KO Hela transfected for 72h with siRNA targeting ATP6V1A or scramble were stained with RagC and Lamp1 antibodies. The graph below represents the colocalization analysis of RagC and LAMP1, expressed as Menders' colocalization coefficient. Results are mean±s.d. n=3 independent experiments. ns= not significant. Tukey's multiple comparisons test.

The interaction of TFEB with active Rags is necessary for mTORC1-mediated phosphorylation and hence inhibition (Martina and Puertollano, 2013; Napolitano *et al.*, 2020). Importantly, co-immunoprecipitation experiments exploiting an exogenous GFP-tagged TFEB construct in FLCN-KO HK-2 cells showed that silencing of the ATP6V1A in FLCN-KO HK-2 resulted in an increased interaction of TFEB with

RagA, RagC and mTOR, both in presence of amino acids and upon Torin treatment (Fig.22). Together these results suggest that silencing of the V-ATPase promotes TFEB phosphorylation and cytosolic retention by increasing mTORC1 lysosomal recruitment and favoring TFEB interaction with the Rags.

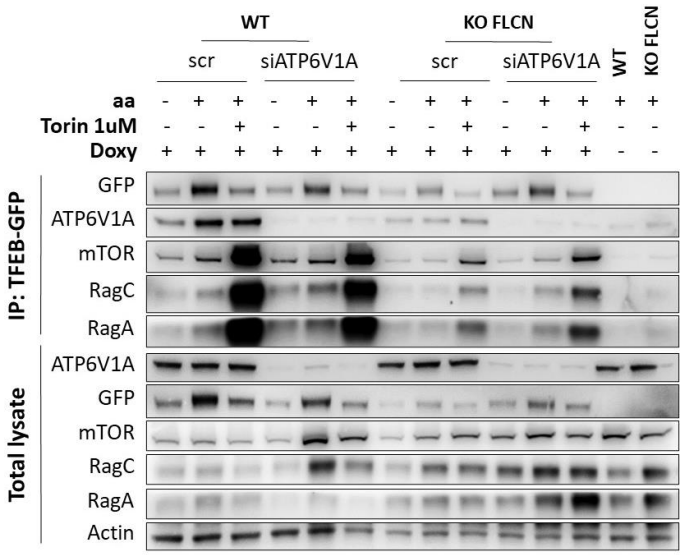
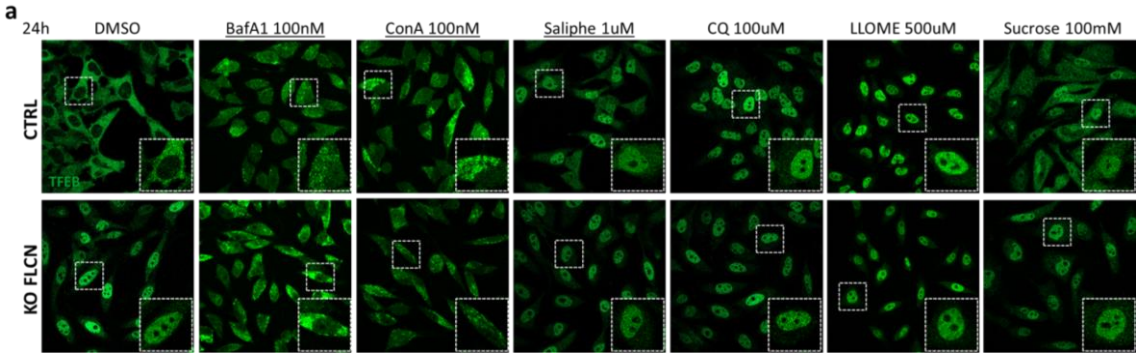


Figure 22. V-ATPase downregulation promotes TFEB binding to the Rags and to mTOR. WT and FLCN-KO HK-2 cells overexpressing TFEB-GFP were transfected with siRNA targeting ATP6V1A or scramble, treated as indicated, incubated with GFP beads and analyzed by immunoblotting for the indicated proteins (WB analysis replicated three times).

7. Pharmacological targeting V-ATPase assembly promotes TFEB cytosolic re-localization, but decreases mTORC1 signaling

The V-ATPase has recently attracted much attention as a potential therapeutic target for cancer and viral infections, owing to its central role in the control of autophagy and intracellular/extracellular pH (Pamarthy *et al.*, 2018; Whitton *et al.*, 2018). This interest has encouraged the study and the synthesis of different inhibitors targeting the V-ATPase by various mean. We investigated the impact of some of these drugs on TFEB translocation in FLCN-KO cells. Surprisingly, we found that long treatment times of FLCN-KO HeLa with the V-ATPase inhibitors BafilomycinA1 (BafA1) or ConcanamycinA (ConA) - both binding the V0c subunit and inhibiting the association of V1 domain with V0 (Huss *et al.*, 2002; Wang *et al.*, 2021) - promote TFEB cytosolic re-localization in presence of nutrients (Fig.23a, 24). This effect is not observed upon treatment with SaliPhe (a different V-ATPase inhibitor that stimulates subunits interaction), with EN6 (an activator of the V-ATPase that uncouples it from Ragulator-Rags complex), and KM91104 (an inhibitor of the V0a3-V1B2 interaction) (Xie *et al.*, 2004; Kartner *et al.*, 2010; Chung *et al.*, 2019) (Fig.23a,b). Eventually, Diphyllin - a drug targeting the V0a2 subunit (Salvi *et al.*, 2022) - has a minor impact on TFEB localization (Fig.23b).



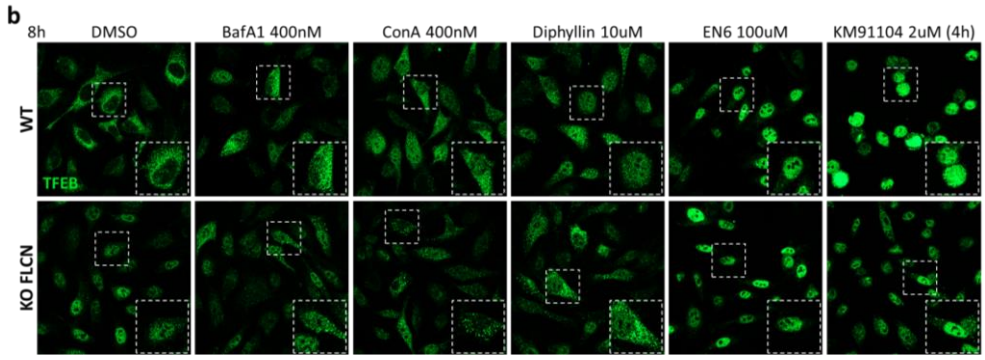


Figure 23. V-ATPase pharmacological inhibition promotes TFEB cytosolic re-localization. (**a**) Immunofluorescence analysis of endogenous TFEB localization in CTRL or FLCN-KO HeLa either untreated (DMSO) or treated for 24h with 100nM Bafilomycin A1 (BafA1) and Concanamycin A (ConA), 1μM SaliPhe, 100μM Chloroquine, 500μM LLOME, and 100mM sucrose. (**b**) Immunofluorescence of TFEB localization in CTRL or FLCN-KO HeLa untreated or treated for 8h with 400nM BafA1 and ConA, 10μM Diphyllin, 100μM EN6, or for 4h with 2μM KM91104.

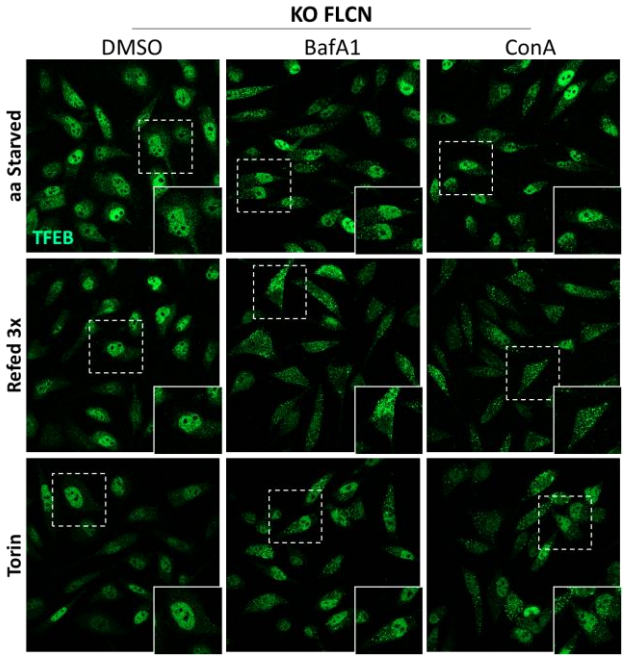


Figure 24. BafA1 and ConA promote TFEB cytosolic re-localization in an amino-acid-sensitive fashion. Immunofluorescent staining of TFEB in FLCN-KO HeLa treated for 24h with 100nM BafA1 or ConA or left untreated (DMSO) and then deprived of amino acids for 1h, or restimulated for 1h the presence or absence of 1μM Torin.

Remarkably, treatment of FLCN-KO cells with BafA1 and ConA downregulates mTORC1 signaling and reduces TFEB protein levels, differently from genetic inhibition of the V-ATPase (Fig.25).

These results suggest that drugs targeting the V-ATPase could be valuable for the treatment of the BHD syndrome, but further studies will be necessary to identify V-ATPase inhibitors that selectively impair TFEB activation.

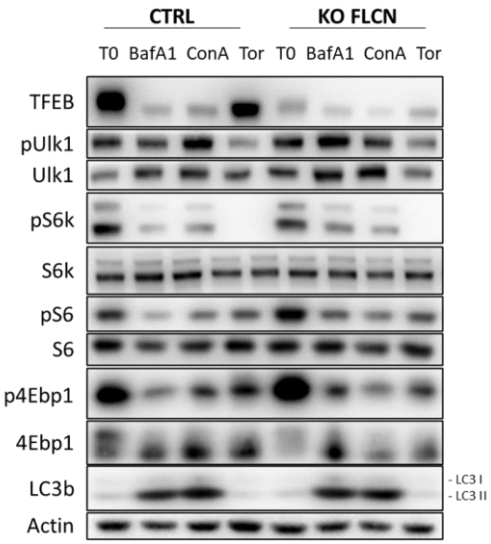


Figure 25. V-ATPase pharmacological inhibition impairs mTORC1 signaling. Immunoblot analysis of the indicated proteins in CTRL or FLCN-KO HeLa cells either untreated (DMSO), treated for 24h with 100nM BafA1 and ConA, or with 1μM Torin for 1h.

8. V-ATPase-mediated TFEB cytosolic retention is independent of lysosomal pH perturbation or xenophagy inhibition

V-ATPase activity ensures organelles pH homeostasis and function (Forgac, 2007; Jansen and Martens, 2012). We wondered if the effect we observed in FLCN-KO cell lines could be linked to changes in lysosomal pH and function, as a consequence of V-ATPase downregulation. We tested some compounds that induce lysosomal alkalization and stress, in particular Chloroquine and LLOME (two lysosomotropic molecules), and sucrose (a lysosomal stressor). Interestingly, these compounds did not correct TFEB nuclear localization in FLCN-KO cells (Fig.23a). These results suggest that the effects of V-ATPase inhibition on TFEB cellular localization are not mediated by the lysosomal stress nor even by alteration of lysosomal pH.

Since MiT/TFE factors are activated by TRPML1-mediated lysosomal calcium efflux (Medina *et al.*, 2015; Di Paola and Medina, 2019), and recent studies suggest that TRPML1 activity and lysosomal pH are strictly interconnected (Li *et al.*, 2017; Xia *et al.*, 2020), one possible scenario was that lysosomal alkalinization mediated by inhibition of the V-ATPase could impair TRPML1-mediated TFEB-activation, thus promoting its cytosolic re-localization. To test this hypothesis, we performed genetic (via siRNA) or pharmacological inhibition of TRPML1 (via the calcium chelator BAPTA-AM) in FLCN-KO cells and evaluated TFEB cellular localization. We observed TFEB nuclear accumulation following these treatments even in response to nutritional availability (Fig.26, 27), thus we concluded that TRPML1 activity is not involved the regulation of TFEB cellular localization mediated by the v-ATPase.

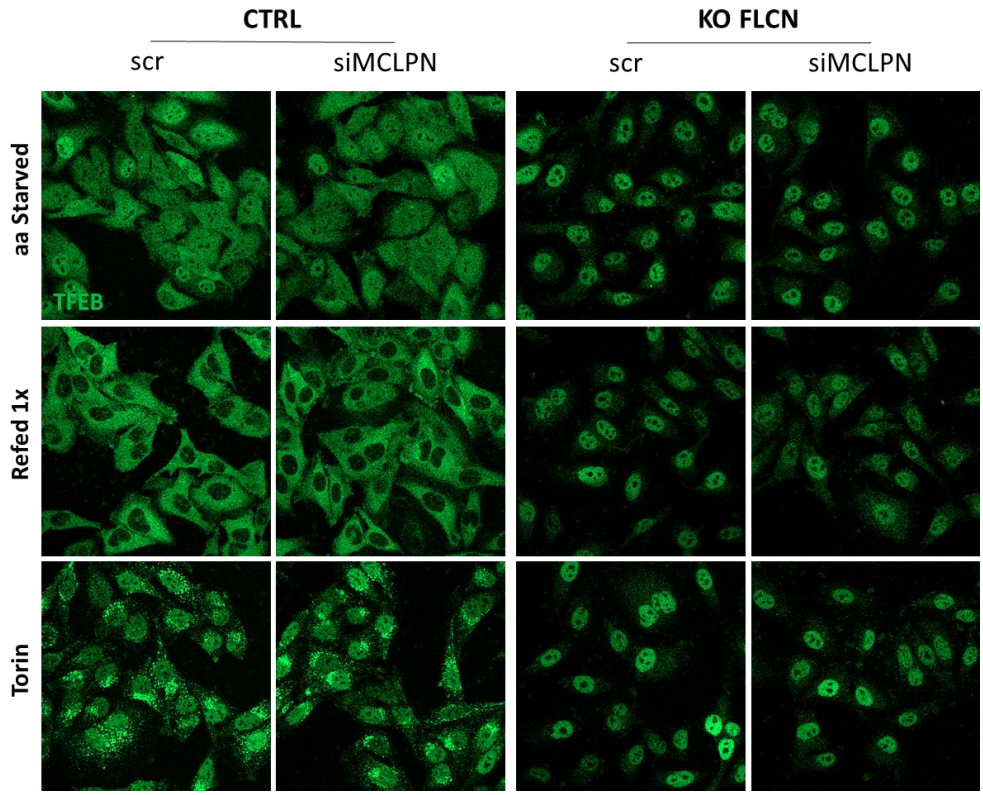


Figure 26. Silencing of TRPML1 does not correct TFEB localization. Immunofluorescence staining of endogenous TFEB in WT and FLCN-KO HeLa transfected for 72h with siATP6V0c or control siRNA, and subjected to either amino acid starvation for 1h, or starvation and refeeding for 1h in the presence or absence of 1μM Torin.

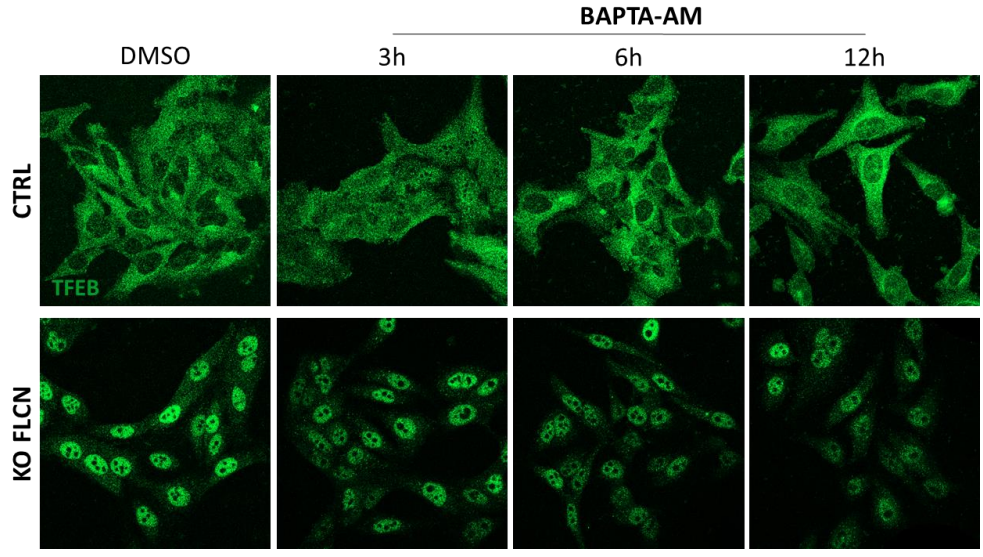


Figure 27. Inhibition of TRPML1 does not correct TFEB localization. Immunofluorescence staining of endogenous TFEB in WT and FLCN-KO HeLa treated with 20μM BAPTA-AM for the indicated time points.

Lysosomal acidification is also fundamental for a proper enzymatic activity of lysosomal hydrolases, and hence in the regulation of autophagy, a catabolic process that efficiently clears cellular cargos. Recently, the Salmonella effector SopF has been identified as a blocker of xenophagy (selective autophagy of intracellular pathogens) by targeting the ATP6V0C (Xu *et al.*, 2019). SopF ADP-ribosylates Gln124 of ATP6V0C and inhibits its recruitment of ATG16L1, thus inhibiting initiation of xenophagy. We asked whether, by inhibiting the interaction of the V-ATPase with ATG16L1, the bacterial SopF could promote TFEB cytosolic re-localization in FLCN-KO HeLa cells. However, disruption of the V-ATPase-ATG16L1 axis by SopF treatment does not rescue TFEB nuclear localization in FLCN-KO cells thus indicating that this pathway is not involved in the V-ATPase-dependent regulation of TFEB activity (Fig.28).

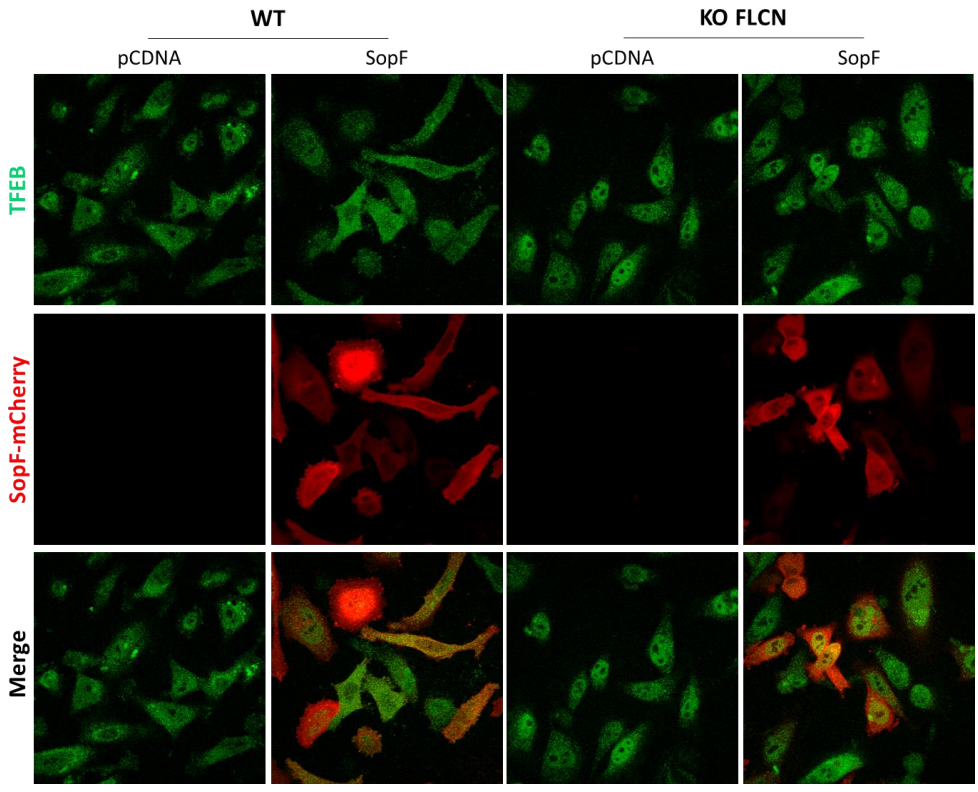


Figure 28. Inhibition of V-ATPase binding to ATG16L1 by SopF does not promote TFEB cytosolic re-localization in FLCN-KO cells. Immunofluorescence staining of endogenous TFEB in WT and FLCN-KO HeLa transfected with empty vector (pCDNA) or SopF-mCherry for 24h.

9. V-ATPase silencing does not impar TFEB-mediated lysosomal damage response

Lysosomal damage and stress trigger TFEB activation in order to initiate lysosomal biogenesis (Chauhan *et al.*, 2016; Lu *et al.*, 2017). Remarkably, treatment with the lysosomotropic drug LLOME selectively inhibits TFEB phosphorylation by mTORC1, without affecting the phosphorylation of the other mTORC1 substrates (Nakamura *et al.*, 2020). Notably, treatments with LLOME succeeded in inducing TFEB nuclear translocation in FLCN-KO cells silenced for ATP6V1A (Fig.29), thus meaning that TFEB-mediated lysosomal damage response is not altered by V-ATPase inhibition.

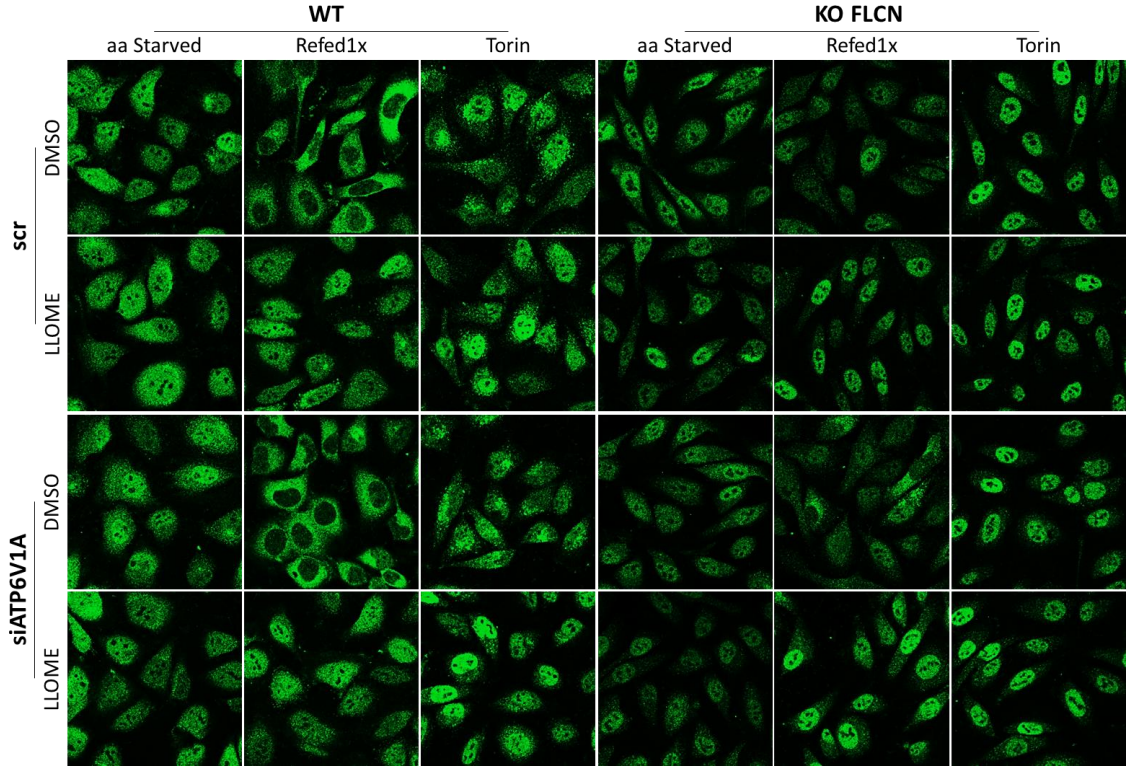


Figure 29. LLOME-induced lysosomal damage activates TFEB in FLCN-KO HeLa silenced for ATP6V1A. Immunofluorescence staining of endogenous TFEB in WT and FLCN-KO Hela transfected for 72h with siRNA targeting ATP6V1A or scramble, then subjected to either amino acid starvation for 1h, or starvation and refeeding for 1h in the presence or absence of 1μM Torin. Cells were treated with 500μM LLOME for 1h.

10. Downregulation of the V-ATPase promotes TFEB cytosolic retention in RagC-KO HeLa cells

To test the role of the Rag GTPases in the regulation of V-ATPase-mediated TFEB cytosolic re-localization, we analyzed HeLa cell lines KO for specific Rags: RagA-KO, RagC-KO, RagC/D-double KO (dKO). In accordance with their essential role in the regulation of mTORC1 signaling and TFEB binding and phosphorylation (Martina and Puertollano, 2013; Napolitano *et al.*, 2020), these cell lines show constitutive TFEB nuclear localization (Fig.30). Intriguingly, we found that ATP6V0C depletion corrects TFEB localization in RagC-KO HeLa cells upon amino acids stimulation, but not in cells lacking RagA or both RagC and RagD (Fig.30). Since Rag-GTPases present different levels of expression, our results suggest that depletion of RagA cannot be compensated by its homologous RagB, whereas RagC depletion can be compensated by RagD.

We have not yet a clear explanation for our findings but a possible scenario is that the silencing of the V-ATPase may, somehow, increases RagC/D activation, thus promoting TFEB phosphorylation and cytosolic re-localization in FLCN-KO cells. Future biochemical studies are needed to validate our hypothesis.

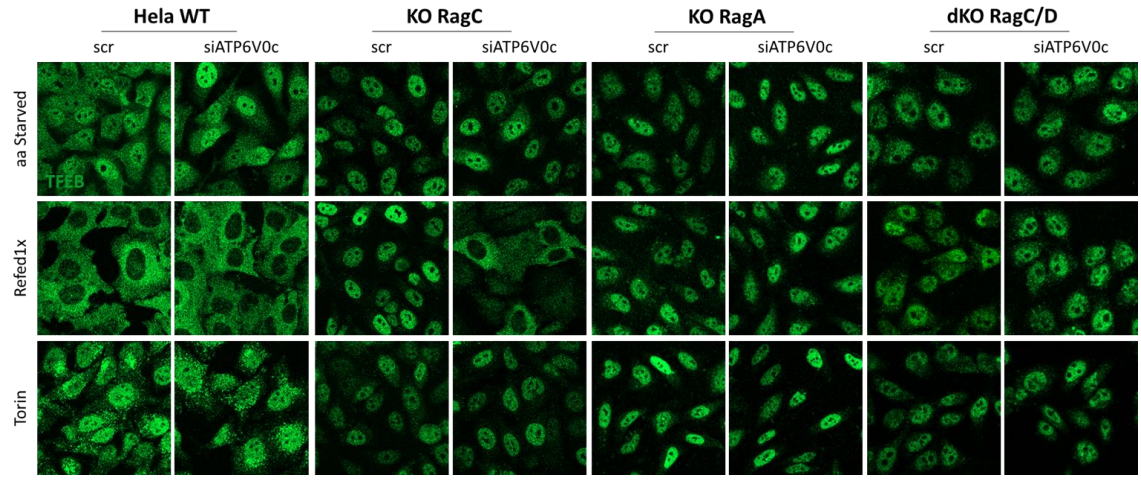


Figure 30. Silencing of the V-ATPase subunits rescues TFEB cytosolic localization in RagC-KO HeLa. Immunostaining of endogenous TFEB in Hela cells WT or KO for *RagC* or *RagA* or *RagC/D* and transfected for 72h with siATP6V0c or control siRNA, and subjected to either amino acid starvation for 1h, or starvation and refeeding for 1h in the presence or absence of 1μM Torin.

11. V-ATPase assembly is enhanced in FLCN-KO cells

Various mechanisms regulate V-ATPase activity in response to different stimuli, first among all is rapid and reversible association of the peripheral V1 domain with the membrane-embedded V0 sector (Forgac, 2007; Cotter *et al.*, 2015; McGuire *et al.*, 2017). It is known that amino acid availability and glucose depletion promotes V-ATPase disassembly (Stransky and Forgac, 2015; McGuire *et al.*, 2017). Furthermore, some drugs inhibiting the V-ATPase modulate its assembly: Bafilomacyn A1 and Concanamycin A prevent V1 association to V0 , while salicylihalamides (SaliPhe) blocks assembled V-ATPase (Huss *et al.*, 2002; Xie *et al.*, 2004; Wang *et al.*, 2021).

Immunoblot analysis of membrane fraction revealed increased levels of the V1A subunit assembled with the V0c in FLCN-KO cells compared to the WT (Fig.31).

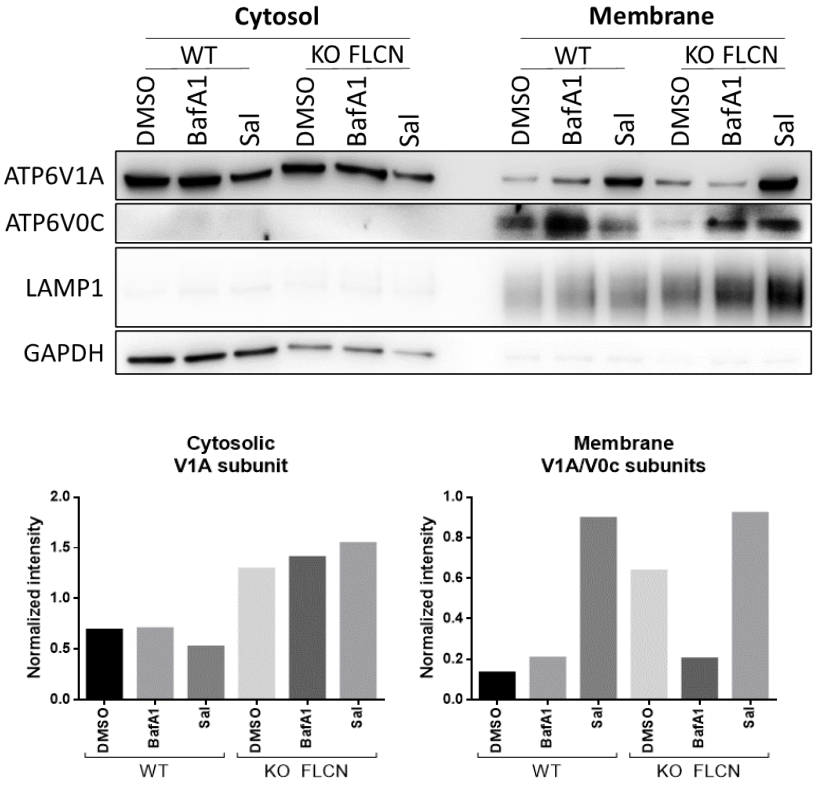


Figure 31. Association of V1 domain with V0 is improved in FLCN-KO cells. Western blot analysis of the indicated proteins in cytosolic and membrane fractions from WT and FLCN-KO HeLa treated for 2h with 200nM Bafilomycin or 2μM SaliPhe. GAPDH and LAMP1 were used as loading control for cytosolic and membrane fractions, respectively.

Moreover, mass spectrometry of immunoprecipitated HA-tagged lysosome revealed enrichment of different V-ATPase subunits in FLCN-KO HeLa compared to WT (Fig.32). These data suggest that the assembly rate of V-ATPase sectors is higher in FLCN-KO cells than in WT.

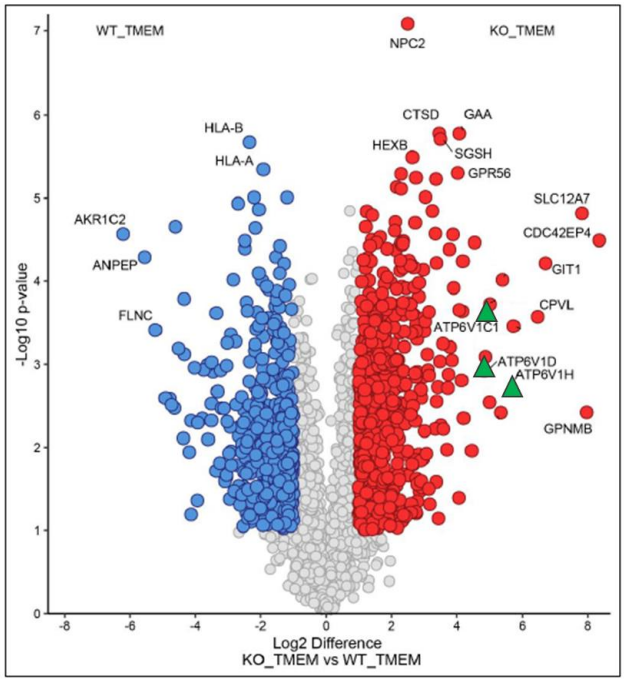


Figure 32. Different V-ATPase subunits are enriched in FLCN-KO lysosomes. Volcano plot depicting some top up- and downregulated genes in lysosomes of FLCN-KO over control cells (n=3). Peptides with fold change log2 ratios >1 or <-1 and -log10 p-value >1 were considered as significantly enriched (labeled in red) or depleted (labeled in blue), respectively. Subunits of lysosomal v-ATPase are labeled in green.

12. The interaction of Ragulator with Rag GTPases and the V-ATPase is strengthened in FLCN-KO cells

The V-ATPase interacts with Ragulator, a pentameric complex that tethers the Rag GTPases to lysosomes, thus promoting mTORC1 activation by amino acids (Zoncu *et al.*, 2011). Amino acid starvation strengthens the interaction of Ragulator with both the V-ATPase and Rag GTPases, while amino acid replenishment weakens these bindings (Zoncu *et al.*, 2011; Bar-Peled *et al.*, 2012). However, the interplay between V-ATPase and Ragulator own many open questions.

Our co-immunoprecipitation analysis showed strong interaction of LAMTOR1, a Ragulator subunit, with RagC and the V1A subunit of the V-ATPase in FLCN-KO cells compared to control cells (Fig.33, 34).

These results, together with the enhanced assembly of V-ATPase (showed above), suggest that an increased association of V-ATPase-Ragulator-Rag GTPases occurs in the context of FLCN deficiency and this may have a role in the regulation of TFEB activity.

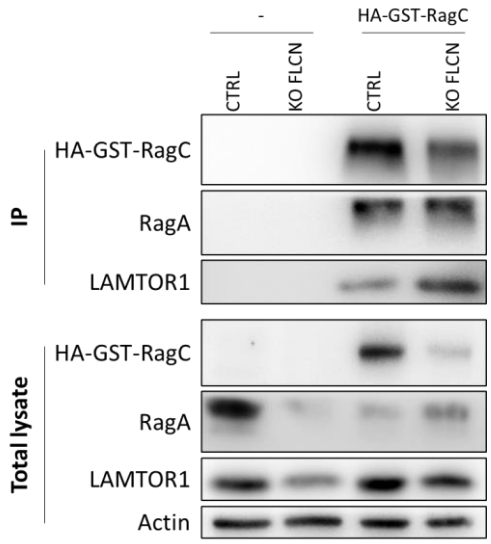


Figure 33. RagC binding with Ragulator is increased in FLCN-KO HeLa. WT and FLCN-KO HeLa cells overexpressing HA-GST-tagged RagC were incubated with HA beads and analyzed by immunoblotting for the indicated proteins.

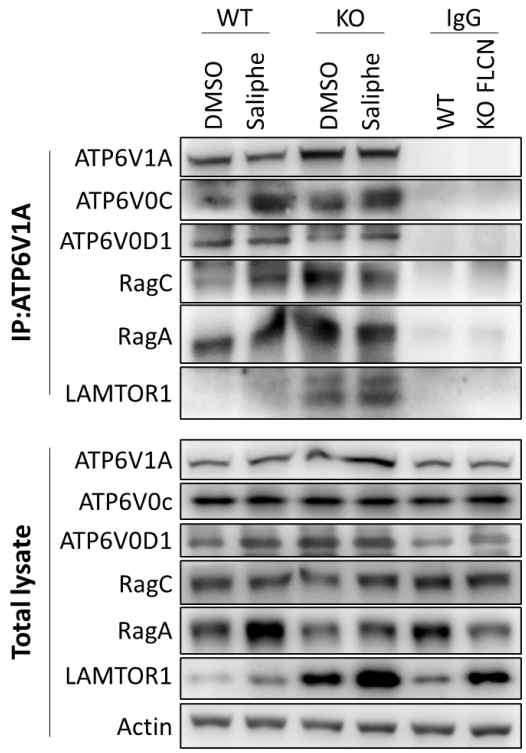


Figure 34. ATP6V1A binding with Rags GTPases and Ragulator is increased in FLCN-KO HeLa. FLCN-KO and WT HeLa were treated with 1μM Saliphe for 2 hours with the aim of blocking V0-V1 assembly of the V-ATPase. Endogenous ATP6V1A from cell lysates was immunoprecipitated with ATP6V1A antibody followed by immunoblotting the indicated proteins.

13. V-ATPase silencing stimulates Ragulator-Rags interaction

Besides its role as a lysosomal scaffold for Rag GTPases and mTOR, Ragulator was also proposed to work as a guanosine nucleotide exchange factor (GEF) for RagA/B, thus promoting their activation (Bar-Peled *et al.*, 2012; Shen and Sabatini, 2018). Anyway, whether the Ragulator complex alone functions as a GEF is still controversial, since other studies showed no measurable GEF activity (Li *et al.*, 2017). Regardless of the impact of Ragulator on Rag GTPases, the Rag-Ragulator complex is essential for the activation of the mTORC1 pathway, and the V-ATPase controls this interaction (Bar-Peled *et al.*, 2012).

We investigated whether downregulation of the V-ATPase could affect the Ragulator-Rag GTPases complex. Although V-ATPase-Ragulator-Rag interaction is already increased in FLCN-KO compared to WT cells, we observed a further increase in the amounts of RagA, RagC and RagD that co-immunoprecipitated with GFP-

tagged LAMTOR1 or Flag-tagged LAMTOR2 after silencing of the V-ATPase in FLCN-KO cells (Fig.35, 36).

Consistent with a possible regulatory role of the V-ATPase for the Rag-Ragulator complex, our findings suggest that depletion of the V-ATPase in cells lacking FLCN could somehow favour Rag GTPase activation by modulating their interaction with Ragulator. However, further studies are needed to evaluate the nucleotide loading state of Rag GTPases in FLCN-KO cells after V-ATPase downregulation, as well as the impact of Ragulator binding in Rag activation.

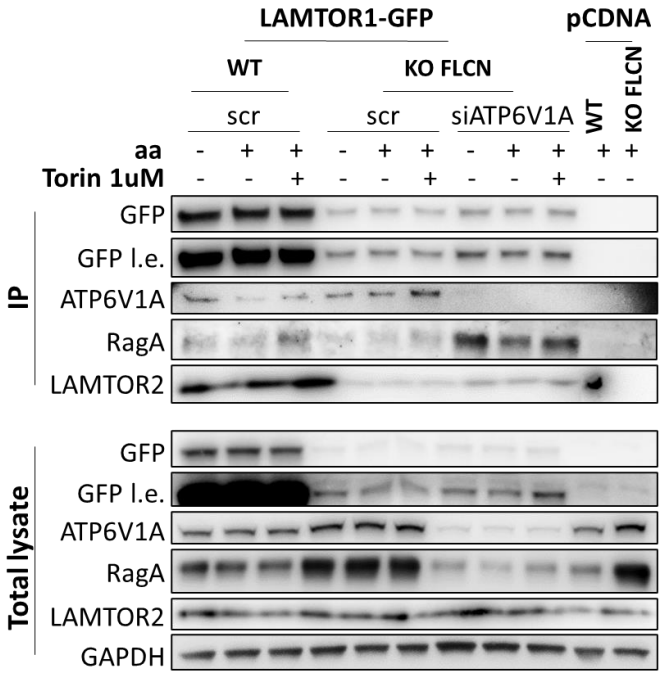


Figure 35. LAMTOR1-RagA binding increases in FLCN-KO cells silenced for ATP6V1A. Western blot analysis of FLCN-KO and WT HeLa transiently expressing GFP-tagged LAMTOR1 or a control plasmid (pCDNA), and transfected with scramble siRNA or siATP6V1A. After 72h of silencing, cells underwent amino acid starvation for 1h, and refeeding for 30min in presence or absence of 1μM Torin. Lysates were immunoprecipitated with GFP beads followed by immunoblotting the indicated proteins.

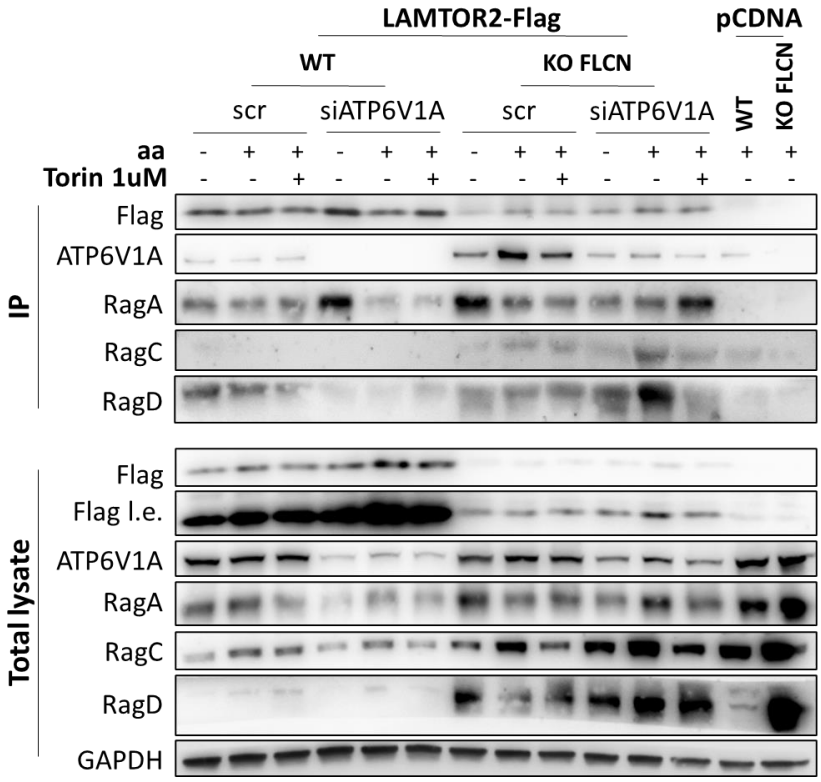


Figure 36. LAMTOR2-RagC/D binding increases in FLCN-KO cells silenced for V1A. Western blot analysis of FLCN-KO and WT HeLa transiently expressing Flag-tagged LAMTOR2 or a control plasmid (pCDNA), and transfected with scramble siRNA or siATP6V1A. After 72h of silencing, cells underwent amino acid starvation for 1h, and refeeding for 30min in presence or absence of 1μM Torin. Lysates were immunoprecipitated with Flag beads followed by immunoblotting of the indicated proteins.

14. Functional Ragulator is required for TFEB cytosolic retention in FLCN-KO cells

Next, we sought to define the role of Ragulator in the regulation of TFEB activity in FLCN-deficient cells. Silencing of the Ragulator subunit *LAMTOR1* induce TFEB nuclear translocation in WT HeLa, in line with the fact that Ragulator serves as an essential scaffold for Rags localization at the lysosome. Notably, silencing of *LAMTOR1* fails to correct TFEB cellular localization in FLCN-KO cells depleted for ATP6V1A subunit, thus suggesting that lysosomal targeting of the Rags is needed to accomplish TFEB phosphorylation and inactivation also in the context of V-ATPase depletion (Fig.37, 38).

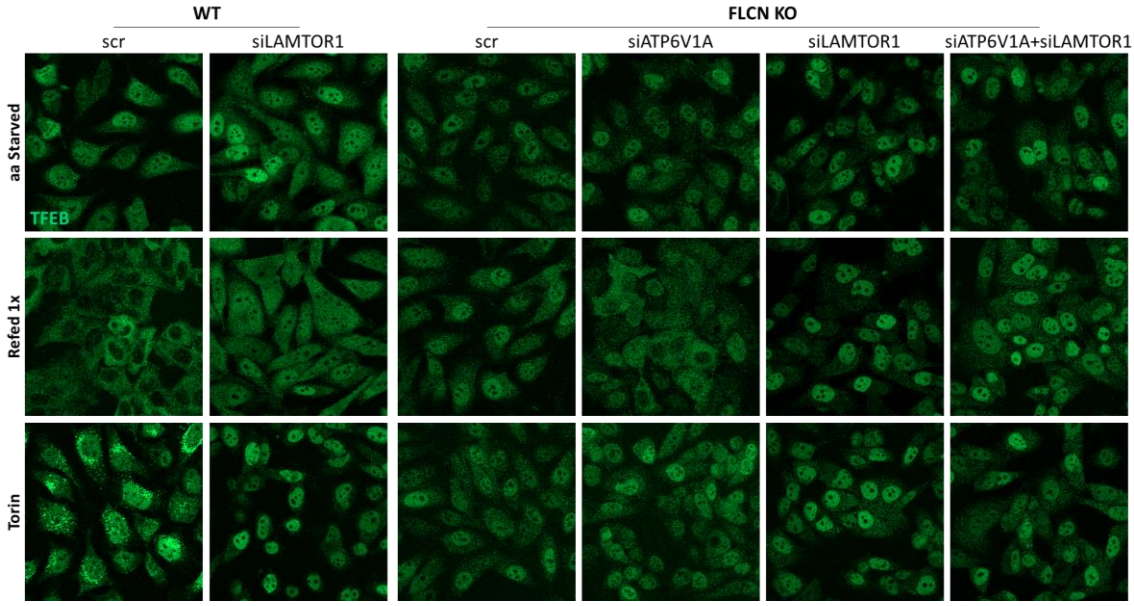


Figure 37. Loss of LAMTOR1 induces TFEB nuclear translocation. Immunofluorescence staining of endogenous TFEB in WT and FLCN-KO HeLa transfected for 72h with siRNA targeting *ATP6V1A*, *LAMTOR1* or both, and subjected to either amino acid starvation for 1h, or starvation and refeeding for 1h in the presence or absence of 1μM Torin.

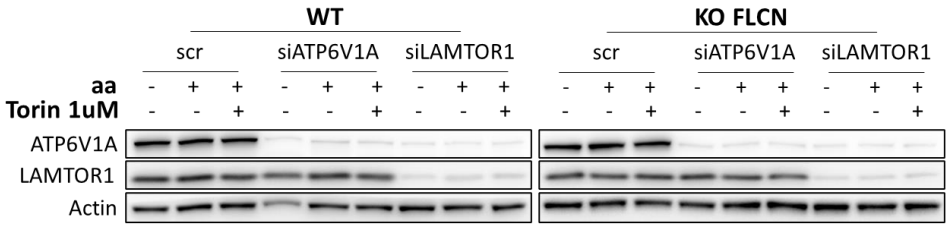


Figure 38. Validation of the silencing of LAMTOR1. Western blot analysis of cells showed in Fig.38, silenced for *ATP6V1A* alone or in combination with *LAMTOR1*.

LAMTOR1 is a 161-amino acid protein that wraps around the other four members of the Ragulator complex and anchors them onto the lysosome thanks to its N-terminal lipidation region (Mu *et al.*, 2017). A large part of LAMTOR1 sequence is involved in the assembly with the other Ragulator proteins (approximately residues 77-156), while the C-terminal tail and the N-terminal α1 helix of LAMTOR1 are required for holding RagA-C onto Ragulator (Yonehara *et al.*, 2017; Zhang *et al.*, 2017). However, very little information is known about the interaction sites of Ragulator with the V-ATPase. A recent paper showed that ubiquitination of the Lysine20 (K20) of LAMTOR1 is required for its binding with the V-ATPase (Hertel *et*

al., 2022). Considering all these data, in order to interfere with V-ATPase-Ragulator binding, we sought to mutate LAMTOR1 N-terminal region including K20, in the following mutants: deletion of the residues 15-35 (Δ15-35), 18-22 (Δ18-22), 20-24 (Δ20-24) and substitution of K20 with Arginine (K20R). Among the mutations examined, all the deletions, but not the Lysine substitution, cause mislocalization of LAMTOR1 away from lysosomes (Fig.39). Unfortunately, none of the mutants rescued TFEB nuclear retention in FLCN-KO cells (insets Fig.39). Further mutational and structural analysis should be conducted to dissect the interaction of the V-ATPase with Ragulator.

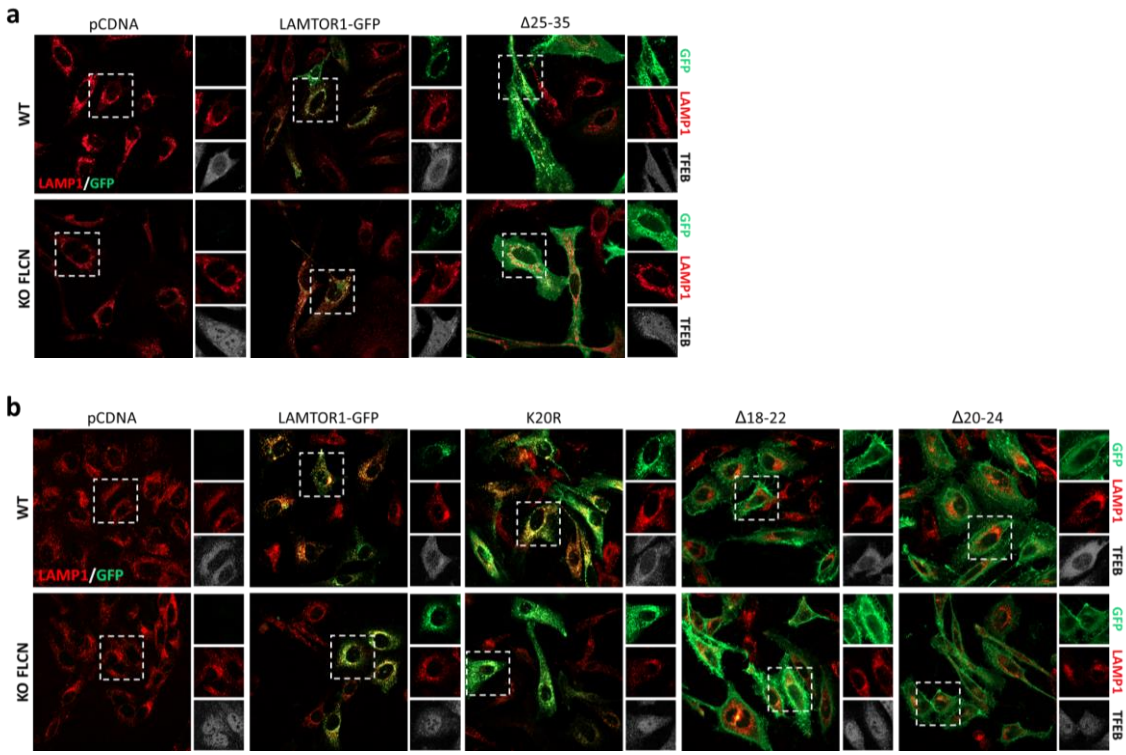


Figure 39. N-terminal mutants of LAMTOR1 delocalize from LAMP1-positive lysosomes and do not correct TFEB nuclear translocation in FLCN-KO cells. Immunofluorescence staining of endogenous LAMP1, TFEB, and GFP-tagged wild-type or mutated LAMTOR1 in WT and FLCN-KO HeLa. Cells were transfected for 24h with the indicated plasmids. TFEB staining of the boxed areas is showed in the insets.

15. V-ATPase downregulation does not rescue TFEB nuclear retention in TSC-KO cells

The BHD syndrome, which is characterized by mutation of *FLCN* and TFEB constitutive activation, is an inherited cancer syndrome associated with the development of kidney cancer (Schmidt and Linehan, 2015; Di Malta *et al.*, 2023). Similarly to the BHD syndrome, the Tuberous Sclerosis Complex (TSC) is caused by loss of function of the tumor suppressors TSC1/2, negative regulators of the mTORC1 pathway, that leads to constitutive activation of TFEB and TFE3, which are the main drivers of tumorigenesis in this disease too (Alesi *et al.*, 2021). We wondered whether downregulation of the V-ATPase was able to correct TFEB subcellular localization in cells lacking TSC2. Interestingly, TFEB remains nuclear in TSC2-KO cells upon silencing of the V-ATPase (Fig.40), thus further suggesting that the V-ATPase-mediated TFEB regulation depends on the FLCN-Rags axis.

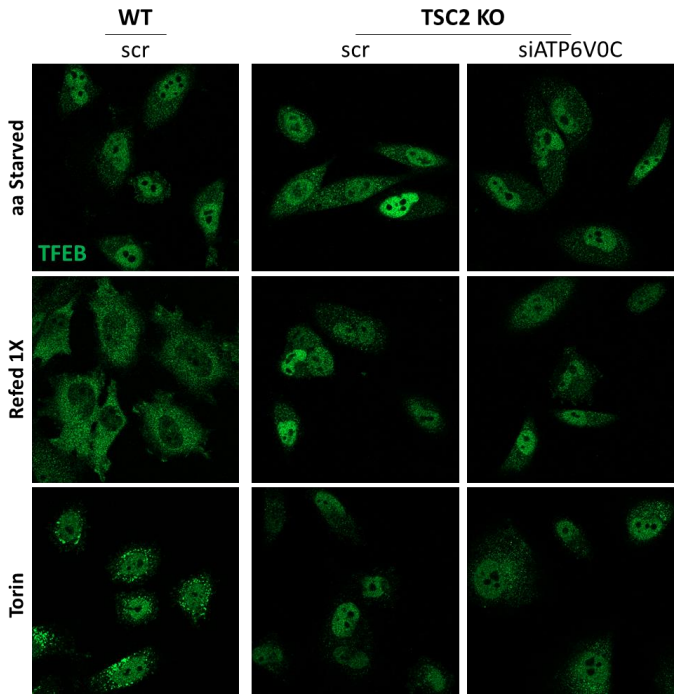


Figure 40. TFEB localization is insensitive to V-ATPase downregulation in TSC2-KO cells. Immunofluorescence staining of endogenous TFEB in WT and TSC2-KO HeLa transfected for 72h with scr or siATP6V0C, and subjected to either amino acid starvation for 1h, or starvation and refeeding for 1h in the presence or absence of 1μM Torin.

DISCUSSION

TFEB and its homologous TFE3 are master regulators of lysosomal biogenesis, activity and signaling (Puertollano *et al.*, 2018; Ballabio and Bonifacino, 2020), and their excessive activation promotes cancer growth (Perera, Di Malta and Ballabio, 2019). In particular, our previous studies clearly indicated that TFEB and TFE3 represent key contributor to kidney cysts and cancer development associated with the Birt-Hogg-Dubé syndrome, a genetic condition due to mutation in the gene encoding FLCN, an essential activator of RagC/D (Napolitano *et al.*, 2020; Di Malta *et al.*, 2023). However, the molecular mechanisms mediating TFEB, as well as TFE3, cellular localization and activity are not fully dissected.

In this study we identified a novel mechanism of regulation of TFEB cellular localization and phosphorylation involving the V-ATPase complex. We found that, under FLCN-deficiency, silencing or pharmacological inhibition of the V-ATPase promoted TFEB and TFE3 cytosolic re-localization in presence of nutrients and this effect was blunted upon treatment with the mTOR inhibitor Torin1 (Fig.10-12, 16-18, 23, 24). In line with these findings, downregulation of the V-ATPase also resulted in increased phosphorylation of TFEB at serine 142, substrate of mTORC1 (Fig.13), and increased binding of TFEB with Rag GTPases and mTOR (Fig.22). In parallel, we also observed decreased expression of different TFEB/TFE3 target genes (Fig.19). Further RNA-seq analysis of FLCN-KO cells silenced for the V-ATPase would be needed to provide a global picture of the transcriptional changes associated with this approach.

Furthermore, we found an increase in the phosphorylation levels of canonical mTORC1 substrates (such as S6K1 and 4E-BP1) upon silencing of the V-ATPase in FLCN-KO cells (Fig.13, 14), and this correlated with increased lysosomal localization of mTOR (Fig.20). On the other hand, the AMPK signaling pathway, which also relies on the V-ATPase activity, was not significantly affected by the downregulation of the V-ATPase in FLCN-KO cells (Fig.15). These results suggest that downregulation of the V-ATPase is influencing the mTORC1 pathway and somehow promoting re-

activation of RagC/D, independent of FLCN-GAP activity. Additional studies aimed at measuring the activation state of Rags heterocomplexes are needed to address this point.

The V-ATPase is fundamental for lysosomal acidification and function; hence its inhibition leads to lysosomal alkalinization and stress (Stevens and Forgac, 1997; Kissing *et al.*, 2015). We treated the FLCN-KO cells with different drugs targeting and impairing the V-ATPase by diverse means: we used L-leucyl-L-leucine methyl ester (LLOME) or Chloroquine, which are lysosomotropic molecules known to induce lysosomal alkalization without affecting the V-ATPase assembly at the lysosome; Bafilomycin (BafA1) or Concanamycin (ConA), both binding to the transmembrane subunit ATP6V0C thus inhibiting the association of V1 with V0; Saliphenylhalamide (SaliPhe), which instead stimulates and block subunit interaction; EN6, that uncouples the V-ATPase from the Ragulator-Rags complex; Diphyllin, that targets the V0a2 subunit and interferes with the proton-pumping action; and KM91104, an inhibitor of the V0a3-V1B2 association. In addition, we treated cells with sucrose, to induce lysosomal stress and see whether this condition could also promote TFEB cytosolic re-localization in FLCN-KO cells. We found that only treatment with BafA1 or ConA promoted TFEB cytosolic re-localization in FLCN-KO cells, thus suggesting that the structural integrity of the V-ATPase, together with the ability to reversible dissociate, are essential to mediate the regulation of TFEB in FLCN-KO cells (Fig.23). This hypothesis was supported by the fact that downregulation of fundamental subunits of the V-ATPase, but not accessory proteins like ATP6AP2, promoted TFEB cytosolic retention (Fig.16).

Along with kinases such as mTOR and AKT, some phosphatases control TFEB phosphorylation state. In particular, the phosphatase Calcineurin de-phosphorylates and activates TFEB in response to calcium release by the lysosomal channel Ca^{2+} TRPML1 (Medina *et al.*, 2015). TRPML1 is subjected to dual regulation

by both Ca^{2+} and pH (Li *et al.*, 2017), thus we reasoned that V-ATPase downregulation could alter TRPML1 activity. By inhibiting and downregulating TRPML1, we ruled out the possibility that silencing of the V-ATPase could impair TRPML1-dependent Ca^{2+} efflux thus inducing TFEB cytosolic retention in FLCN-KO cells (Fig.26, 27). We also excluded the hypothesis that V-ATPase-ATG16L1 interaction, and hence xenophagy pathway, could be involved in the V-ATPase-mediated TFEB re-phosphorylation and cytosolic re-localization in FLCN-KO cells, by exploiting the transient transfection with the xenophagy inhibitor SopF (Fig.28).

The V-ATPase, along with Ragulator and Rag GTPases, participates to the amino acid sensitive recruitment of mTORC1 to the lysosomal membrane (Zoncu *et al.*, 2011; Zhang *et al.*, 2014). Notably, we found that the binding of Ragulator to the Rag GTPases and the V1A subunit of the V-ATPase was enhanced in FLCN-KO compared to WT cells (Fig.33, 34), and silencing of the V-ATPase further increased association of Ragulator to the Rags (Fig. 35, 36). Our hypothesis is that the high lysosomal assembly of the V-ATPase (Fig.31, 32) may somehow exert an inhibitory effect on Rag GTPases' activity in absence of FLCN by affecting Ragulator-Rags interaction. In order to specifically interfere with the V-ATPase-Ragulator association, without altering the correct Ragulator lysosomal localization, we tried to generate LAMTOR1 mutants unable to bind the V-ATPase but still competent to localize at the lysosome and bind with the Rags. However, our attempt failed because all the LAMTOR1 mutants that we generated so far showed impaired lysosomal localization (Fig.39). More work is needed to explore this hypothesis.

Interestingly, a similar correction of TFEB nuclear localization by the V-ATPase was observed in HeLa cells knocked out for RagC but not in cells depleted of RagA or both RagC and RagD (Fig.30). It is possible that the lack of RagC can be compensated by RagD in response to V-ATPase depletion, whereas RagB levels are too low to compensate for the lack of RagA. However, these results are in line with

the idea that the V-ATPase-dependent regulation of TFEB activity in FLCN-KO cells is dependent on the Rags.

Notably, silencing of the V-ATPases did not promote TFEB cytosolic re-localization in a cellular model of Tuberous Sclerosis Complex (Fig.40), a genetic condition associated with mTORC1 hyperactivation and TFEB constitutive activation, similar to BHD (Alesi *et al.*, 2021).

Importantly, the V-ATPase is indispensable for cell viability, indeed knocking out any of the V-ATPase genes cause cell death (Xu *et al.*, 2019). Therefore, our investigation was hampered by the lack of a stable model of V-ATPase downregulation. Since, as expected, our attempts to generate KO cell lines for ATP6V1A gene failed, we tried to exploit heterozygous cell lines, but we observed that heterozygous mutations of the ATP6V1A gene as well as the short hairpin RNA-mediated downregulation of this subunit were unable to significantly decrease ATP6V1A protein levels (data not shown).

In summary, our study identifies a new mechanism of regulation of TFEB activity mediated by the V-ATPase under FLCN deficiency, and dependent on mTORC1 function. Our results support the hypothesis that the V-ATPase, or some of its interactors, could be responsible for the putative "re-activation" of RagC/D in absence of FLCN, still acting through Ragulator. However, additional players may be involved in this mechanism and further studies are needed to fully shed light on this novel pathway.

Remarkably, different V-ATPase inhibitors are available and our work could encourage further studies exploiting these molecules as a therapeutic alternative for the treatment of BHD-dependent tumors.

MATERIALS AND METHODS

Materials

Antibodies were obtained from the following sources: human TFEB, phospho-Ser211 TFEB, TFE3, phospho-Ser757 ULK1, ULK1, phospho-Thr389 S6K1, S6K1, phospho-Ser240/244 S6, S6, phospho-Ser65 4E-BP1, 4E-BP1, mTOR, RagA, RagC, RagD, FLCN, FNIP2, LAMTOR1, phospho-Ser79 ACC, ACC, phospho-Thr172 AMPK, AMPK from Cell Signaling Technology; phospho-Ser142 TFEB from Merck Millipore; GFP, FNIP1, ATP6V0D1, ATP6V1A, ATP6V1D and ATP6AP2 from Abcam; GAPDH and LAMP1 from Santa Cruz Biotechnology; Actin and Flag from Sigma Aldrich; PARP1 from Enzo Life Sciences; HA from BioLegend; LC3 from Novus Biologicals.

MEM, glutamine, Penicillin/Streptomicin and Fetal Bovine Serum (FBS) were from Euroclone. DMEM/F12, ITS and OptiMEM were from ThermoFisher Scienific. Dialyzed FBS, Alexa 488, 594 and 647-conjugated secondary antibodies, lipofectamine LTX and lipofectamine RNAimax were from Invitrogen. RPMI without amino acids and glucose was from US Biological Life Sciences. Amino acids and polybrene were from Sigma Aldrich.

Torin 1, Bafilomycin A1, EN6, KM91104, Diphyllin, Chloroquine, Doxycycline, Puromycin and DMSO were from Sigma Aldrich. LLOME and Concanamycin A were from Santa Cruz Biotechnologiy. BAPTA-AM and Blasticidin were from ThermoFisher Scienific. SaliPhe was kindly provided by J. Goodwin.

The following beads and resins were used for immunoprecipitation experiments: anti-GFP trap agarose beads were from Chromotek; anti-Flag M2 beads were from Sigma Aldrich; Pierce Anti-HA Agarose, Pierce Anti-HA Magnetic Beads and Dynabeads Protein G were from ThermoFisher Scienific.

Complete Protease Cocktail and phosphatase inhibitors (Phospho Stop tablets) were from Roche. MOPS powder and mPAGE™ 4-12% bis-tris gels were from Merck Millipore. Vectashield Antifade Mounting Medium with DAPI was from Vector Laboratories.

Cell cultures

Cells were cultured at 37 °C and 5% CO_2 in the following media supplemented with 10% inactivated FBS, 2mM glutamine and Penicillin/Streptomicin 100µg/ml: HeLa in MEM and HK-2 in DMEM/F12 supplemented with ITS (Insulin-Transferrin-Selenium).

FLCN-KO, RagA-KO, RagC-KO, RagC/D-dKO, TSC2 KO cell lines were generated using the CRISPR–Cas9 system and FACS-sorted into 96-well plates to obtain single-cell-derived colonies carrying the indel mutations.

HK-2 cells with inducible expression of TFEB-GFP were generated upon transduction of these cells with pLVX-TetONE-GFP-TFEB inducible lentiviral plasmid. HeLa cells stably expressing TMEM192-3xHA or HA-GST-RagC were generated upon transduction respectively with pLJC5-Tmem192-3xHA and pLJC6-HA-GST-RagC lentiviral plasmids.

All cell lines were purchased from ATCC, and routinely tested for absence of mycoplasma

Plasmids

pLVX-TetONE-GFP-TFEB inducible lentiviral plasmid was previously generated in our laboratory (Sambri *et al.*, 2023). pJMI-FLAG-LAMTOR2 was a gift of C. Settembre. pLJC5-Tmem192-3xHA, pLJC6-HA-GST-RagC, N1-p18/LAMTOR1-EGFP and pmCherry-SopF were purchased from Addgene. N1 constructs for expression of GFP-tagged LAMTOR1 mutants K20R, Δ18-22 and Δ20-24 were generated by using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies), while GFP-LAMTOR1(Δ25-35) was generated using the In-fusion HD cloning kit (Takara).

Cell treatments and protein knockdown

For experiments involving amino acid starvation, cells were rinsed twice with PBS and incubated for 60 minutes in amino acid-free RPMI supplemented with 10%

dialyzed FBS. For amino acid refeeding, cells were re-stimulated for 30-60 minutes with 1x or 3x water-solubilized mix of essential and non-essential amino acids resuspended in amino-acid-free RPMI supplemented with 10% dialyzed FBS, plus glutamine. Torin treatment was performed with 1μM Torin1 during restimulation.

For drug treatment experiments, cells were incubated for 24 hours in medium containing the following compounds: BafilomycinA1 (BafA1) 100nM, ConcanamycinA (ConA) 100nM, SaliPhe 1μM, Chloroquine (CQ) 100μM, L-Leucyl-L-Leucine methyl ester hydrobromide (LLOME) 500μM, Sucrose 100mM. Cells were also treated for 8 hours with the following drugs: BafA1 400nM, ConA 400nM, Diphyllin 10μM, EN6 100μM, and KM91104 2μM for 4 hours (8h-treatment resulted cytotoxic). TRPML1 inhibition was achieved treating cells with 20μM BAPTA-AM for 3, 6 and 12 hours. In order to monitor TFEB-mediated lysosomal damage response, cells were treated with 500μM LLOME for 1 hour. Untreated cells were incubated with DMSO or water, accordingly to the solubility of the drugs used in the experiments.

For siRNA-based experiments, cells were transfected in OptiMEM using Lipofectamine RNAiMAX with 40nM of the indicated siRNA and analyzed after 72h. The following siRNAs were used: siRNA ATP6V0D1, ATP6V1A, ATP6V1D, ATP6AP2, MCLPN and non-targeting siRNA were SMARTpool from Dharmacon; siRNA ATP6V0C was from Ambion; siRNA Lamtor1 was from Sigma Aldrich.

Plasmids were transfected with Lipofectamine LTX in OptiMEM using a reverse transfection protocols and, 48h upon transfection, cells were analyzed by immunofluorescence or immunoblotting. For mCherry-SopF immunofluorescence, cells were transfected in 6-well dishes with 1μg of pmCherry-SopF plasmid. For immunoprecipitation analysis, cells were transfected in 150mm dishes with 16ug of pJMI-FLAG-P14/LAMTOR2 or N1-p18/LAMTOR1-EGFP. Empty pcDNA3 plasmid was HK-2 cells transduced with the TetONE-GFP-TFEB plasmid were treated with 2,5μg/ml Doxycycline for 24 hours in order to induce the expression of TFEB-GFP.

Mammalian lentiviral production and transduction

Mammalian lentiviral production was produced by transfection of HEK293T cells with lentiviral plasmids (pLVX-TetONE-GFP-TFEB, pLJC5-Tmem192-3xHA, pLJC6-HA-GST-RagC) in combination with the pCMV-VSV-G and pCMV-DVPR packaging plasmids using Lipofectamine LTX transfection reagent. Medium was changed 24h after transfection to DMEM supplemented with 10% FBS. Virus-containing supernatants were collected 48h later, passed through a 0.45µm filter to eliminate cell debris and used for infection of HeLa or HK-2 cell lines in the presence of 5µg/ml polybrene at a multiplicity of infection (MOI) 1. Cells were selected with puromycin (TFEB-GFP HK-2 and TMEM192-3xHA HeLa) or blasticidine (HA-GST-RagC HeLa) 48h after infection.

Cell lysis and western blotting

Cells were rinsed once with PBS and lysed in ice-cold lysis buffer (250mM NaCl, 1% Triton, 25mM Hepes pH 7.4) supplemented with protease and phosphatase inhibitors. Total lysates were passed 10 times through a 25-gauge needle with syringe, kept at 4°C for 10 min and then cleared by centrifugation in a microcentrifuge (14'000 rpm at 4°C for 10 min). Protein concentration was measured by BCA assay.

All cell lysates were resolved by SDS-polyacrylamide gel electrophoresis on 4–12% Bis-Tris gradient gels and analyzed by immunoblotting with the indicated primary antibodies.

Co-immunoprecipitation

For immunoprecipitations of tagged proteins, cells grown in 150mm culture dishes were washed twice with warm PBS and then incubated with 1mg/ml DSP crosslinker for 7 min at room temperature (RT). The crosslinking reaction was quenched by adding Tris-HCl pH8.5 to a final concentration of 100mM. Cells were rinsed once with ice cold PBS and lysed with lysis buffer (40mM Hepes pH7.4, 2mM EDTA, 1%

NP-40, 1% sodium deoxycholate and 0.1% SDS) supplemented with proteases and phosphatase inhibitors. 1mg of cell lysate was incubated with anti-GFP trap agarose beads (for TFEB-GFP and LAMTOR1-GFP), M2-Flag beads (for LAMTOR2-FLAG) or HA-Agarose at 4°C overnight on a rotating wheel, washed five times, and eluted in Laemmli buffer for immunoblotting analysis.

For immunoprecipitation of endogenous ATP6V1A, cells were plated in 150mm dishes and treated for 2 hours with 1μM Saliphe or DMSO. Cells were rinsed twice with cold PBS, and lysed with lysis buffer (150mM NaCl, 20mM TrisHCl pH7,5, 1% Np40) supplemented with proteases and phosphatases inhibitors. Total lysates were passed ten times through a 26-gauge needle with syringe, kept at 4°C for 10 min and then cleared by centrifugation 14'000 rpm at 4°C for 10 min. 2μg of ATP6V1A antibody (abcam) or IgG (as control) per 1mg of lysate were conjugated to 250μg of Dynabeads Protein G in PBS with 0,1% Tween20 for 10 minutes on a rotating wheel, then magnetic beads were washed once with PBS-Tween20. After antibody binding, 1mg of lysate was incubated with the conjugated magnetic beads overnight on a rotating wheel at 4°C. Finally, magnetic beads were washed five times in the lysis buffer and eluted in Leammli buffer for western blot analysis.

Lysosome-Immunopurification (Lyso-IP)

Cells plated on 150mm dishes were rinsed once with PBS and collected in PBS. After a centrifugation at 1'000rpm for 10 minutes in a 4°C centrifuge, pellets were resuspended in sub-fractionation buffer (SB) (140mM KCl, 250mM Sucrose, 1mM DTT, 2mM EGTA, 2,5mM MgCl2, 25mM HEPES, pH 7.24) supplemented with protease inhibitor, then centrifuged at 1'000g for 2 minutes at 4°C. Cells were lysed in 500μl of SB by using a grind pestle. 1,5mg of lysate was incubated with anti-HA Magnetic Beads for 45 minutes at 4°C on a rotating wheel. Subsequently, beads were washed ten times with SB, but for the last two washes SB was supplemented with 300mM NaCl. Immunoprecipitated lysosomes were eluted with 0,5% Np40 in SB.

Nucleus/cytosol fractionation

FLCN-KO HeLa cells were plated on 150mm plates and transfected with non-targeting siRNA and siATP6V1A. After 72h, cells were subjected to amino acid starvation and refeeding treatments as described above. Then cells were rinsed and collected in PBS and lysed in buffer A (20mM TrisHCl pH7.4, 0,1mM EDTA, 2mM $MgCl_2$) supplemented with protease and phosphatase inhibitors. 1% NP40 was added later in the vials to protect nuclei from a premature lysis, then cell pellets were passed 3 times through a 20-gauce needle. After a brief centrifugation at 500g, the supernatant containing the cytosolic fraction was collected and further purified by three centrifugations of 10 minutes with increasing speed (2'000, 6'000 and 14'000rpm). In addition, the pellet containing nuclei was washed three times with buffer A plus 1% NP40, then it was resuspended in buffer B (20mM Hepes pH7.4, 400mM NaCl, 1mM EDTA, 0,5mM DTT). Nuclei were subjected to four cycles of flash-freezing in liquid nitrogen and thawing in a 37°C bath, then left to thaw in ice for about 20 minutes. Finally, the nuclear lysate was centrifuged for 20 minutes at 20'000g. The supernatant was the nuclear fraction.

Organelle/cytosol fractionation

Cells were plated on 150mm plates and left untreated or treated for 2 hours with 100nM BafA1 or 2μM SaliPhe. After drug treatments, they were rinsed twice 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 centrifugation at 1'700rpm for 10 minutes, pellets were resuspended in 750ul of FB and lysed by 23G needle; subsequently 750ul more of FB was added to lysates, then it was centrifuged at 2'700rpm 10 min in a 4°C centrifuge. Post-nuclear supernatant (PNS) was transferred to a clean tube and ultracentrifuged at 100'000g for 20min. The supernatants were the organelle-free cytosolic fractions. The pellets (organelles)

were washed twice with FB and eluted in Laemmli buffer. Equal fractions of pellets and supernatants were analyzed by western blot.

Mass spectrometry (MS) and MS data analysis

All the experiments were performed in a labeling free setting. Proteins from Lyso-IP were precipitated in acetone overnight at -20°C, reduced and alkylated in a solution of 6M guanidine-HCl, 5mM tris(2-carboxyethyl)phosphine (TCEP), and 20mM chloroacetamide, then digested with LysC (Wako) for 3 hours at 37°C and with the endopeptidase sequencing-grade trypsin (Promega) overnight at 37°C. Collected peptide mixtures were concentrated and desalted using the Stop and Go Extraction (STAGE) technique (Rappsilber, Ishihama and Mann, 2003).

MS data acquisition and analysis was performed by the Mass spectometry facility at Tigem. Instruments for LC-MS/MS analysis consisted of a NanoLC 1200 coupled via a nano-electrospray ionization source to the quadrupole-based Q Exactive HF benchtop mass spectrometer. Peptide separation was carried out according to their hydrophobicity on a home-made chromatographic column, 75 µm ID, 8 Um tip, 250mm bed-packed with Reprosil-PUR (C18-AQ), 1.9µm particle size, 120Angstrom pore size, using a binary buffer system consisting of solution A (0.1% formic acid) and B (80% acetonitrile, 0,1% formic acid). Runs of 75 min were used for Lyso-IP, with a constant flow rate of 300nl/min. MS data were acquired using a data-dependent top-15 method with maximum injection time of 20ms, a scan range of 300–1650Th, an AGC target of 3e6 and a resolution of 120,000. Resolution, for MS/MS spectra, was set to 45,000 at 200 m/z, AGC target to 1E5, maximum injection time to 20ms and the isolation window to 1.4Th. The intensity threshold was set at 2.0 E4 and Dynamic exclusion at 30s.

Raw MS data were processed with MaxQuant (1.6.2.10) using default settings (FDR = 0.01, oxidized methionine and acetylation as variable modifications, and carbamidomethyl as fixed modification). For protein assignment, spectra were correlated with the Uniprot Homo Sapiens database (v.2019), including list of

common contaminants. Bioinformatics analysis was performed with Perseus 1.6.2.363. The label-free quantification intensities were logarithmized, grouped and filtered for minimum valid number (min. three in at least one group). Missing values have been replaced by random numbers that are drawn from a normal distribution. Proteins with Log2 ratios ≥ 1 and a p-value ≤ 0.05 were considered significantly enriched. To identify significant enriched GO terms in Lyso-IP, we utilized the 1D enrichment tool in Perseus. The protein-protein interaction network was built in the Cytoscape environment. Proteins belonging to the selected cluster were loaded into the STRING plugin and the network was subsequently generated.

Immunofluorescence and confocal microscopy

Cells were fixed in PFA 4% for 15 min and permeabilized with blocking buffer 0,02% saponin and 3% bovine serum albumin in PBS for 1 hour at RT. For endogenous TFEB and TFE3 immunostaining cells were permeabilized in 0.1% Triton X-100 for 5 minutes before blocking buffer incubation to favour visualization of the nuclear signal. Cells were incubated with the indicated primary antibodies in the blocking buffer 2 hours at RT, except for TFEB and TFE3 staining that were incubated overnight at 4°C; subsequently cells were incubated with secondary Alexa-Fluor conjugated secondary antibodies for 1 hour at RT. Cells were finally mounted in Vectashield mounting medium with DAPI and analyzed using LSM800.

For confocal imaging, the samples were examined under a Zeiss LSM800 confocal microscope with 63x oil immersion objective.

Images were processed in ImageJ and Mender's colocalization coefficients were calculated using JACoP ImageJ plugin.

For high content screening analysis, the images were acquired with an automated confocal microscopy and analyzed through Columbus Image Data Storage and Analysis System. A dedicated script was applied to evaluate TFEB nuclear translocation.

RNA extraction, reverse transcription and quantitative PCR

RNA samples from cells were obtained using the RNeasy kit (Qiagen) according to the manufacturer's instructions. 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 minutes; (95°C, 10s; 60°C, 10s; 72°C, 15s) × 40. Fold change values were calculated using the ΔΔCt method. Internal control HPRT1 was used as 'normalizer' gene to calculate the ΔCt value. Next, the ΔΔCt value was calculated between the 'control' group and the 'experimental' group. Finally, the fold change was calculated using 2(−ΔΔCt).

Statistical analysis

Statistical analyses were performed using GraphPad Prism8.0. Two-way ANOVA and Tukey's or Sidak's post hoc tests were performed to determine significant differences between groups and interactions between the two factors. $P < 0.05$ was considered significant.

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