Amino acid sensory complex proteins in mTORC1 and macroautophagy regulation

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Abstract

Autophagy is the highly conserved catabolic process, which enables the survival of a cell under unfavorable environmental conditions. In a constantly changing environment, cells must be capable of dynamically oscillating between anabolism and catabolism in order to maintain cellular homeostasis. In this context, the activity of the mechanistic Target Of Rapamycin Complex 1 (mTORC1) is of major importance. As a central signaling node, it directly controls the process of macroautophagy and thus cellular metabolism. Thereby, the control of mTORC1 is equally crucial as the regulation of cellular homeostasis itself, whereby particular importance is attributed to amino acid sensory proteins. In this review, we describe the recent findings of macroautophagy and mTORC1 regulation by upstream amino acid stimuli in different subcellular localizations. We highlight in detail which proteins of the sensor complexes play a specific role in this regulation and point out additional non-canonical functions, e.g. in the regulation of macroautophagy, which have received little attention so far.

Introduction

To maintain cellular homeostasis, proliferating cells exhibit a high demand for energy and building blocks. However, within the cellular microenvironment a constant supply of nutrients may not always be ensured. In order to adapt to this constantly changing environment, cells must possess the utmost metabolic plasticity and be capable to recycle existing intracellular components as a source both for anabolic processes as well as for adenosine triphosphate (ATP) production. A central mechanism that controls and maintains this metabolic plasticity is the highly conserved catabolic process of autophagy, which also promotes cell survival in response to various stress conditions, e.g. nutrient deficiencies. By catabolizing dysfunctional molecules, such as protein aggregates, and cell organelles via lysosomal degradation, autophagy plays an invaluable role not only in maintaining healthy cellular homeostasis and development but also in preventing many diseases, including cancer [1]. A central complex for the fine-tuned adaptation of autophagy is the mechanistic Target Of Rapamycin Complex 1 (mTORC1), a critical signaling node that is conserved from yeast to humans, and regulates both cell growth and metabolic homeostasis by integrating diverse environmental cues such as growth factors, nutrients, energy levels and stress (reviewed e. g. in [2–4]). Within the last decade, many proteins have been identified to play a role in nutrient sensing mechanisms, in particular of amino acids, upstream of mTORC1 and thus of autophagy [4–8].

The aim of this review is to summarize advances on macroautophagy regulation by amino acid sensory and mTORC1-regulatory proteins. We will

cover what is known about the functions of the individual proteins of this network, and their impact on mTORC1-dependent and -independent aspects of macroautophagy regulation.

Autophagy

There are three types of autophagy described: (i) macroautophagy, (ii) microautophagy and (iii) chaperone-mediated autophagy (CMA). Macroautophagy (often referred to as 'autophagy') is the best described process, which involves the sequestration of bulk of cytoplasmic content in double-membrane vesicles followed by the fusion and subsequent degradation within lysosomes. Macroautophagy can be subdivided into non-selective ('bulk autophagy') or selective (e.g. the degradation of damaged organelles such as mitochondria/mitophagy as well as invading bacteria/xenophagy). Although macroautophagy varies in terms of target specificity or induction conditions, it uses a common molecular core machinery as described in the next paragraph. In contrast, in microautophagy, smaller portions of the cytoplasm are directly entrapped by lysosomal and endosomal membrane dynamics and made accessible for degradation. In addition, microautophagy, in comparison to macroautophagy, exhibits a much more diverse morphology and molecular mechanisms [9,10]. In CMA, degradation of a specific cargo bearing a C-terminal pentapeptide KFERQ motif is selectively mediated by the Heat Shock Cognate Protein of 70 kDa (HSC70), and directly delivered to lysosomes for degradation without the formation of any additional vesicular structures [11,12]. However, in the further course of this review we will focus on the process of macroautophagy.

As depicted in Fig. 1, the core processes of macroautophagy can be divided into five main steps: (1) phagophore initiation followed by (2) elongation of phagophores, (3) phagophore closure, (4) maturation of autophagosomes and fusion with lysosomes and finally (5) degradation and recycling of the autophagic cargo. In response to various stimuli, e.g. metabolic stress such as amino acid scarcity, macroautophagy initiation leads to the formation of a crescent-shaped membrane structure, referred to as the phagophore, at specific phagophore assembly sites (PAS), which can be found at various organelles, e.g. the endoplasmatic reticulum (ER) and the Golgi apparatus [1,13]. The initiation and thus the formation of the phagophore involves the activation of the UNC-51-Like Kinase (ULK) complex and the Class III Phosphatidylinositol 3-Kinase (PI3K) complex. The ULK complex consists of ULK1 or its paralogue ULK2, Autophagy-Related Gene (ATG) 13, ATG101, and Focal Adhesion Kinase (FAK)-Family Interacting Protein (FIP200). Several studies and a recent computer-based analysis showed that ULK1 and ULK2 are less redundant than previously thought. Despite its enzymatic similarity to ULK1, ULK2 differs in its autophagy-related interactors and their post-translational and transcriptional regulators [14]. The PI3K complex consists of the core complex, composed of Vacuolar Protein Sorting (VPS) 15 and VPS34, also known as PI3KC3, as well as Beclin-1 (BECN1), which interacts with ATG14L and Activating Molecule In Beclin-1-Regulated Autophagy (AMBRA1) to form the subcomplex PI3KC3-Complex 1 (PI3KC3-C1) [15]. Following activation of the ULK complex, ULK1 phosphorylates and activates PI3KC3-C1 [16]. The resulting VPS34 lipid kinase activity produces a pool of phosphatidylinositol 3-phosphate (PI(3)P) on the membrane of the phagophore, which recruits the effector proteins WD Repeat Domain Phosphoinositide-Interacting Proteins (WIPI, e.g. WIPI2) and Zinc-Finger FYVE Domain-Containing Protein 1 (DFCP1). Consequently, inhibition of VPS34 inhibits macroautophagy [17]. During the elongation of the phagophore, two ubiquitin-like protein conjugation systems are of primary importance, namely the ATG12-ATG5-ATG16L complex and the ubiquitin-like ATG8 protein family (Light Chain 3 (LC3) subfamily: LC3A, LC3B, LC3C; γ-Aminobutyric Acid Receptor-Associated Protein (GABARAP) subfamily: GABARAP, GABARAP-Like 1 (GABARAPL1) and GATE-16/GABARAPL2. It has been demonstrated that through the interaction of the effector protein WIPI2 with ATG16L the ATG12-ATG5-ATG16L complex is recruited to the PAS [18]. This enables a series of enzymatic reactions leading to the conjugation of ATG8 family proteins to phosphatidylethanolamine (PE) resulting in the membrane bound lipidated forms (ATG8 conjugation reaction) [19]. Furthermore, the sequestration of specific cargo is mediated by the ATG8-PE's interaction with autophagy receptors (selective macroautophagy, [20,21]). For phagophore closure, and thus the formation of double-membrane vesicles called autophagosome, the study of Takahashi *et al.* could show for the first time that proteins of the Endosomal Sorting Complex Required For Transport (ESCRT) machinery are central for this process. Especially the loss of the ESCRT-III complex protein Charged Multivesicular Body Protein 2A (CHMP2A) led to an accumulation of phagophores and an impairment of the autophagic flux. Similar observations were made for VPS4, important for the depolymerization of ESCRT-III assemblies, and the ESCRT-I subunit VPS37A, which is essential for the recruitment of, e.g., CHMP2A [22,23]. For a detailed description on autophagosome biogenesis see the recent review of Melia *et al.* [24].

Upon autophagosome formation, the maturation of the autophagosome and its outer membrane fusion with the lysosome leads to the formation of the autophagolysosome, in which more than 60

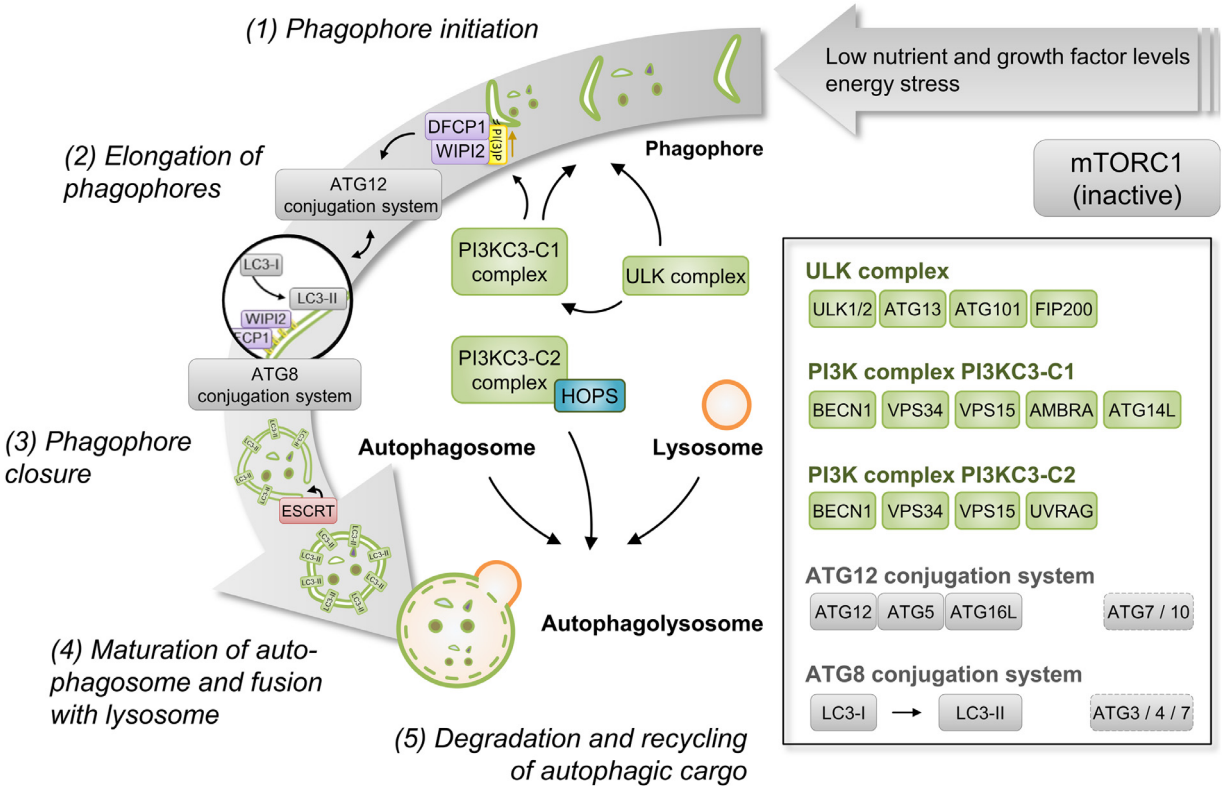

Fig. 1. Macroautophagy initiation during cellular stress – Macroautophagy can be categorized into five main steps: (1) Phagophore initiation by active ULK complex, which in turn triggers the activation of PI3KC3-C1 and nucleation of the phagophore. PI3KC3-C1 activates local PI(3)P production, followed by WIPI2 and DFCP1 recruitment. (2) Elongation of phagophores by the recruitment of two ubiquitin-like conjugation systems (ATG12- and ATG8-conjugation system). ATG7 and ATG10, which function as E1- and E2-like enzymes, respectively, facilitate ATG12 to ATG5 conjugation, which binds ATG16L. Recruitment of the E3-like ATG12-ATG5-ATG16L1 complex by WIPI2 enhances the ATG3-mediated conjugation of ATG8 (LC3-I) family members (LC3-A, -B and -C, as well as GABARAP, GABARAPL1 and GABARAPL2) to membrane-based lipidated forms (ATG8-PE/ LC3-II) – a characteristic feature of autophagic membranes. Previous ATG4-mediated cleavage of newly synthesized pro-LC3 family members enables conjugation to PE. (3) Phagophore closure is facilitated by the ESCRT machinery. (4) The maturation of autophagosomes and fusion with lysosomes involves PI3KC3-C2, which recruits the HOPS-autophagosome-lysosome-tethering complex. (5) Degradation of autophagic cargo by acidic hydrolases leads to recycling of cellular components (e.g. amino acids).

Abbreviations: AMBRA, Activating Molecule In Beclin-1-Regulated Autophagy; ATG, Autophagy-Related Gene; BECN1, Beclin-1; DFCP1, Double FYVE Domain-Containing Protein 1; E1, Ubiquitin-Activating Enzyme; E2, Ubiquitin-Conjugating Enzyme; E3, Ubiquitin Ligase; ESCRT, Endosomal Sorting Complexes Required For Transport; FIP200, Focal Adhesion Kinase (FAK)-Family Interacting Protein 200; GABARAP, γ-Aminobutyric Acid Receptor-Associated Protein; HOPS, Homotypic Fusion And Protein Sorting-Tethering Complex; LC3, Light Chain 3; mTORC1, mechanistic Target Of Rapamycin Complex 1; PE, phosphatidylethanolamine; PI(3)P, phosphatidylinositol (3)-phosphate; PI3KC3-C1/2, Class III Phosphatidylinositol 3-Kinase Complex 1/2; ULK1/2, Unc-51 Like Autophagy Activating Kinase 1/2; UVRAG, UV Radiation Resistance-Associated Gene Protein; VPS, Vacuolar Protein Sorting-Associated Protein; WIPI2, WD Repeat Domain Phosphoinositide-Interacting Protein 2.

known hydrolytic enzymes digest the inner membrane of the autophagosome as well as the enclosed cargo. This allows the generation of cellular building blocks such as amino acids, lipids and nucleotides to be transported back into the cytoplasm and recycled for cellular metabolism. This process entails the involvement of another PI3K subcomplex (PI3KC3-C2). The PI3KC3-C2 subunit UV Radiation Resistance-Associated Gene Protein (UVRAG) binds and positively regulates the Homotypic Fusion And Vacuole Protein Sorting (HOPS)-tethering complex [25,26]. The HOPS complex in turn facilitates membrane fusion by tethering autophagosomes to lysosomes through interaction with the autophagosomal soluble N-Ethylmaleimide-Sensitive Factor Attachment Protein Receptor (SNARE) protein Syntaxin-17 (STX-17) [26,27]. The Protein Associated With UVRAG As Autophagy Enhancer (PACER) serves as a positive regulator of this process by interacting with STX-17 to recruit the HOPS complex to autophagosomes [28]. The final fusion is then achieved by the interaction of proteins on the respective membranes of the autophagosomes and lysosomes (e.g. ADP-Ribosylation Factor-Like

Protein 8B (ARL8b)^{GTP} and Ras-Related Protein RAB-7 (RAB7)^{GTP}). For more details on this process, see the recent reviews of Yim and Mizushima and Lawrence and Zoncu [29,30].

Macroautophagy and its regulation by mTORC1

In addition to its function as a terminal organ within the autophagy process, lysosomes, in close interplay with the essential serine/threonine kinase mTORC1 - composed of mTOR, Regulatory-Associated Protein Of mTOR (RAPTOR), Mammalian Lethal With SEC13 Protein 8 (mLST8), Proline-Rich AKT Substrate Of 40 kDa (PRAS40) and DEP Domain Containing mTOR Interacting Protein (DEPTOR) - act as the central regulator of macroautophagy. Thereby, the cellular metabolic performance is directly reflected in the activity of mTORC1. Thus, mTORC1 acts as an integrator of various upstream signals such as the presence of growth factors, amino acids, energy or changes in cellular redox homeostasis and triggers downstream anabolic processes from the lysosomal surface, which lead to growth at both cellular and organismal level [3,29,30]. Concurrently, mTORC1 activity directly controls macroautophagy by inhibiting various autophagic steps including the initiation and elongation of phagophores, as well as the maturation of autophagosomes.

Regarding the induction of macroautophagy, mTORC1 modulates the kinase activity of ULK1/2 in a nutrient- and growth factor-dependent manner, thereby inhibiting phagophore initiation under nutrient-rich conditions [31]. Here, mTORC1 prevents the activation of the ULK1 complex by phosphorylating ULK1 at S757 and ATG13 at S258 [31,32]. Furthermore, by targeting components of PI3KC3-C1, mTORC1 inhibits (i) the lipid kinase activity of VPS34 by phosphorylation of various residues on ATG14L (S3, S223, T233, S383 and S440) [33] and (ii) the recruitment of PI3KC3-C1 to the ER membrane and thus macroautophagy induction by phosphorylation of AMBRA at S52 [34]. An additional component of the PI3K complex is Nuclear Receptor-Binding Factor 2 (NRBF2), which has been described to be an mTORC1 target and a fine-tuning regulator that promotes autophagic flux when dephosphorylated [35]. Under nutrient-rich conditions, phosphorylation of NRBF2 at S113 and S120 increases its binding affinity to VPS34-VPS15, thereby inhibiting lipid kinase activity [35].

In addition, the process of phagophore elongation to form the autophagosome was reported to be controlled by mTORC1-dependent phosphorylation of WIPI2 and lysine acetyltransferase P300. Thereby, the phosphorylation of WIPI2 at S395 promotes binding of the E3 ligase HECT, UBA and WWE Domain Containing E3 Ubiquitin Protein Ligase 1 (HUWE1) and subsequent ubiquitin-mediated degradation of WIPI2 [36]. P300, known to acetylate histone and non-histone proteins, was identified as an mTORC1-activated target that, when phosphorylated, acetylates LC3 and prevents LC3 lipidation to LC3-PE (LC3-II) [37]. Furthermore, the influence of mTORC1 on autophagosome maturation was described through the regulation of the proteins UVRAG and PACER. When nutrients are available and mTORC1 is active, PACER is phosphorylated at S157, which impedes interaction with the HOPS complex and prevents autophagosome maturation [38]. Regarding UVRAG, phosphorylation at S498 leads to increased affinity for Run Domain Protein As Beclin 1 Interacting And Cysteine-Rich Containing (RUBICON), which inhibits HOPS activation, and subsequently prevents autophagosome maturation and fusion with the lysosome [39,40].

Although inhibition of mTORC1 is necessary to induce and maintain macroautophagy, mTORC1 activity can also serve to promote autophagic flux. During autophagic lysosome reformation (ALR), a cellular process to restore free lysosome levels to maintain lysosome homeostasis during the final phase of autophagy [41,42], mTORC1 has to be reactivated. In this context, mTORC1 phosphorylates UVRAG at S550 and S571, which activates the lipid kinase activity of VPS34-UVRAG complex resulting in regeneration of proto-lysosomal tubules to protect starved cells against cell death [43]. Altogether, by the differential regulation of certain PI3K complexes, mTORC1 inhibits autophagosome biogenesis, but also maintains the autophagic flux by recycling autolysosomal membranes to ensure cell survival during starvation.

Moreover, while the key role of mTORC1-mediated autophagy inhibition is attributed to its kinase activity to phosphorylate certain proteins of the core machinery of macroautophagy, mTORC1 has also been shown to transcriptionally regulate macroautophagy. Several members of the Microphthalmia/Transcription Factor E (MiT/TFE) family have been shown to bind Coordinated Lysosomal Expression And Regulation (CLEAR) responsive elements in the promoters of several macroautophagy-associated genes (BECN1, WIPI1, ATG9B, NRBF2, GABARAP, LC3B, ATG5, Sequestosome-1 (SQSTM1/p62), UVRAG, VPS11, VPS19 and RAB7), thereby controlling the expression of these genes, which led to the identification of Transcription Factor EB (TFEB) as a master regulator of macroautophagy [44,45]. Mechanistically, the activity of these transcription factors is regulated by phosphorylation-dependent control of subcellular localization. Under nutrient-rich conditions, TFEB is recruited to lysosomes, where it is phosphorylated by mTORC1. Phosphorylated TFEB binds to 14-3-3 protein and is thus retained in the cytoplasm. In the case of nutrient deficiency, the

associated inactivation of mTORC1 results in an accumulation of unphosphorylated TFEB in the nucleus and transcriptional activation of macroautophagy genes [46]. Nutrient repletion leads to phosphorylation of TFEB by mTORC1 in conjunction with Glycogen Synthase Kinase 3 Beta (GSK3b), resulting in active export from the nucleus via Exportin 1 (XPO1) [47].

Regulation of mTORC1 by the nucleotide loading state of RHEB and RRAG GTPases

A central feature in the regulation of mTORC1 activity is the amino acid-dependent recruitment of mTORC1 to the surface of lysosomes [48] and the regulation of the nucleotide loading state of the GTPases RAS Homolog Enriched In Brain (RHEB) as well as of the RAS-Related GTP Binding Proteins (RRAGs) [49,50] (Fig. 2). On the lysosomal surface, mTORC1, which is otherwise auto-inhibited by PRAS40 [51], is allosterically activated by the GTPase $RHEB^{GTP}$. It should be noted that the activation of mTORC1 by RHEB cannot only take place on the surface of lysosomes, since RHEB has also been detected on the surface of other organelles such as the Golgi apparatus, the peroxisome, the plasma membrane and the ER [52–55]. This strongly suggests that inter-organelle communication and localization (e.g. Golgi apparatus and lysosomes) are of essential importance for the activation of mTORC1 [30,56]. As implicated in Fig. 2A, the activity of $RHEB^{GTP}$ is inhibited by the trimeric Tuberous Sclerosis Complex (TSC), a GTPase-Activating Protein (GAP) for RHEB (Fig. 2A), which is composed of TSC1, TSC2 and TBC (TRE-2/BUB2/CDC16) Domain 1 Family Member 7 (TBC1D7). The GAP activity of the complex is ascribed to TSC2, whereby TSC1 is required to stabilize TSC2 and to prevent its ubiquitin-mediated degradation [57,58]. TBC1D7 interacts with TSC1-TSC2. Its TBC domain, which has GAP activity toward certain members of the RAB protein family in other TBC proteins, may suggest that TBC1D7 is also bound to other small G proteins, thus implicating that the TSC complex has additional cellular functions beyond the regulation of RHEB [59]. It is generally accepted that environmental cues such as growth factor deprivation and

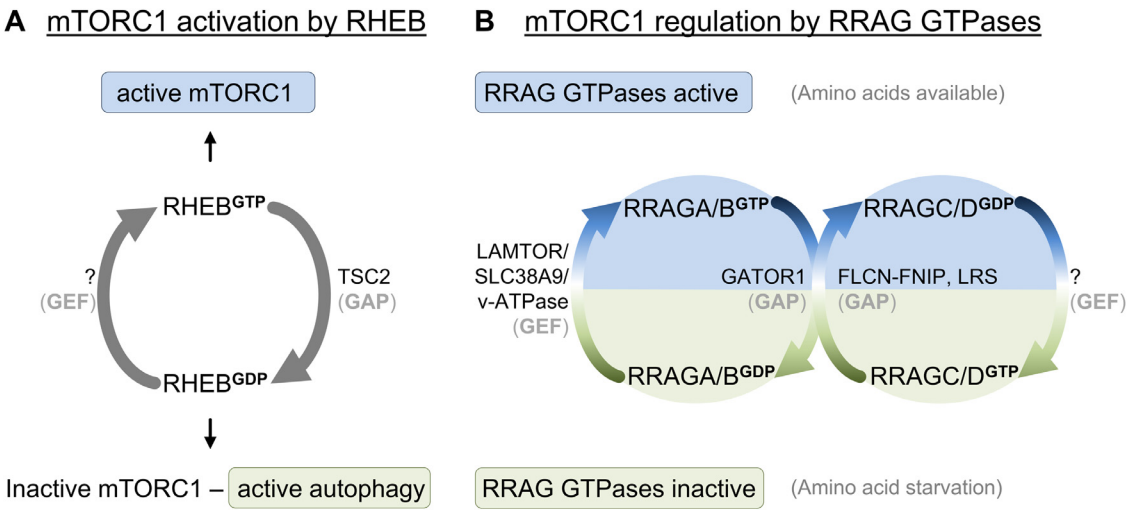

Fig. 2. mTORC1 activation by RHEB and RRAG GTPases A) Active $RHEB^{GTP}$ activates mTORC1. GTP hydrolysis to $RHEB^{GDP}$ by TSC2 GAP activity leads to mTORC1 inactivity, which promotes autophagy. The GEF towards RHEB remains elusive. B) After repletion of amino acids, RRAG GTPases are in their active conformation and mTORC1 is translocated to the lysosomal membrane where $RHEB^{GTP}$ activates mTORC1. Prerequisites for mTORC1 localization are the GEF activity towards RRAGA/B by Ragulator/LAMTOR complex (consisting of LAMTOR1-5, with LAMTOR 4 and 5 being most important in mTORC1 regulation) and the GAP activity towards RRAGC/D by the Folliculin complex (FLCN-FNIP) and LRS. Upon arginine binding, SLC38A9 interacts with the Ragulator complex, converting RRAGA to the active GTP-bound state. Additional GEF-activity exhibit Ragulator/LAMTOR-v-ATPase interaction. By interaction with LRS, leucine directly promotes GAP activity towards RRAGD. Depletion of amino acids activates GATOR1, which exhibits a GAP activity towards RRAGA/B leading to the inactive conformation of RRAG GTPases ($RRAGA/B^{GDP}$). The GEF towards RRAGC/D is not known.

Abbreviations: FLCN, Folliculin; FNIP, Folliculin Interacting Protein; GAP, GTPase-Activating Protein; GDP/GTP, guanosine di-/ triphosphate; GATOR, GAP Activity Towards RRAG; GEF, Guanosine Triphosphate Exchange Factor; LAMTOR, Late Endosomal/Lysosomal Adaptor And MAPK And mTOR Activator; LRS, Leucyl-tRNA Synthetase; mTORC1, mechanistic Target Of Rapamycin Complex 1; RHEB, RAS Homolog Enriched In Brain; RRAGA/B/C/D, Ras Related GTP Binding A/ B/ C/ D; SLC, Solute Carrier; TSC2, Tuberous Sclerosis Complex 2.

low energy converge on TSC. However, several recent studies have nicely demonstrated that also amino acid deprivation leads to lysosomal recruitment of TSC2, clearly indicating a direct control of mTORC1 by TSC within the amino acid sensing pathway. Thus, TSC serves an integration point that inhibits mTORC1 at the lysosomal membrane in response to various inhibitory signals [60–62].

Further, to bring RHEB into close proximity with mTORC1, mTORC1 is recruited to lysosomes via a RRAG-dependent mechanism. In the presence of amino acids, mTORC1 interacts with the pentameric Ragulator/LAMTOR (LAMTOR1 (p18), LAMTOR2 (p14), LAMTOR3 (MP1), LAMTOR4 (HBXIP) and LAMTOR5 (C7orf59))-RRAG complex, which is tethered to the lysosomal membrane. Mammals express four RRAG GTPases – RRAGA, RRAGB, RRAGC and RRAGD, which form heterodimers with RRAGA or RRAGB pairing with RRAGC or RRAGD. The RRAG heterodimers exist in an active and inactive conformation dependent on their nucleotide loading state [63,64]. RRAG heterodimers have four possible nucleotide-binding states, which are regulated by GAPs and Guanine Nucleotide Exchange Factors (GEFs) (Fig. 2B). In the presence of amino acids, the active conformation (RRAGA/B^{GTP} and RRAGC/D^{GDP}) binds RAPTOR and mTORC1 is recruited to the lysosomal membrane [65,66]. The RRAG heterodimers are often used synonymously in the literature, but the publication of Anandapadamanaban *et al*. [42] demonstrated the importance of the RRAGA/C heterodimer in binding the mTORC1 subunit RAPTOR. Moreover, the RRAGB/D heterodimer has been suggested to have an initiating and dominant function over RRAGA/C in mTORC1 activation with respect to leucine signaling [43]. Accordingly, a precise role of specific RRAG heterodimers remains to be elucidated [67,68]. *In vitro* and *in vivo* binding assays have indicated that RRAGC^{GDP} but not RRAGA^{GTP} governs the RAPTOR-RRAG GTPase interaction. GTP loading of RRAGC abolishes the strong interaction with RAPTOR [69]. Mechanistically, RAPTOR was shown to bind RRAGA^{GTP} transiently followed by complete binding to RRAG GTPases when RRAGC is GDP-bound [66,67].

With regard to macroautophagy, RRAG-dependent regulation of mTORC1 was shown to be essential for neonatal macroautophagy in *RRAGA$^{GTP/GTP}$* knock-in mice, as measured by the number of autophagosomes and autophagolysosomes, lipidation of LC3B, and cytoplasmic retention of the transcription factor TFEB [70].

Fully bound to lysosomes by RRAG GTPases, mTORC1 is activated by RHEB, inhibits autophagy and promotes cell growth. It is important to note that complete activation of mTORC1 at lysosomes is only achieved if both growth factors and nutrients are available ('AND circuit') [7]. Although, an 'AND circuit' is described for the activity of RHEB GTPase and RRAG GTPase [3], it should be mentioned, that under conditions of metabolic homeostasis, thus in the presence of growth factors and amino acids, it was shown that the recruitment of mTORC1 can also be independent of RRAG GTPase [60,71]. For instance, another small GTPase, ADP Ribosylation Factor 1 (ARF1), traditionally known to be involved in Golgi membrane trafficking and to be an activator of Phospholipase D (PLD), has been shown to be involved in glutamine-dependent, RRAG GTPase-independent mTORC1 recruitment to and activation at lysosomes. Furthermore, this activity was shown to require Vacuolar Type ATPase (v-ATPase) but not the Ragulator/LAMTOR complex [71]. Thus, small GTPases seem to play a central role in mTORC1 activation, even though they fulfill distinct functions depending on which amino acid is sensed.

In contrast, during amino acid deprivation, RRAG GTPases are in their inactive conformation (RRAGA/B^{GDP} and RRAGC/D^{GTP}) and preferentially bind to the Ragulator/LAMTOR complex, preventing the recruitment of mTORC1 to lysosomes and thus its close proximity of RHEB [72–75]. Furthermore, a recently published study was also able to provide evidence that in response to growth factor or amino acid restriction, the RRAG GTPase (RRAGA/C) drives rapid cycling of TSC between lysosomes and the cytosol. Once recruited, TSC also prevents mTORC1 activation [62]. A further level of complexity is achieved by the fact that for the inactivation of RRAG GTPases the GAP Activity Towards RRAG 1 (GATOR1) complex is required, leading to an exchange of GTP to GDP in the RRAGA/B nucleotide binding pocket. Initially, the GATOR1 complex was described together with the GATOR2 complex as a multiprotein complex, however, it has been shown that in mammalian cells an interaction between GATOR1 and GATOR2 in cells is only weakly detectable or not detectable at all dependent on the cell line tested [74,76,77]. It is however accepted that GATOR1 acts as a GAP for RRAGA/B, thereby inhibiting mTORC1, and GATOR2 in turn inhibits GATOR1 via a so far unknown mechanism [5,74,75,78]. As depicted in Fig. 3, GATOR1 consists of three proteins (Nitrogen Permease Regulator 2 and 3-Like Protein (NPRL2 and 3) and DEP Domain-Containing 5 (DEPCD5)) [74,79]. Structurally, DEPDC5 binds to RRAGA/C and is bound to NPRL3 via NPRL2 [80], whereby it was also shown that in cells lacking DEPDC5, NPRL2/3 are associated with RRAGA/C. From a functional point of view, it has been demonstrated that the highly conserved arginine R78 within the sequence of NPRL2 fulfills GATOR1's GAP function towards RRAGA [81]. Furthermore, the analysis of the structure of GATOR1 has led to the development of a two-state model of the GATOR1 function. In this model the strong

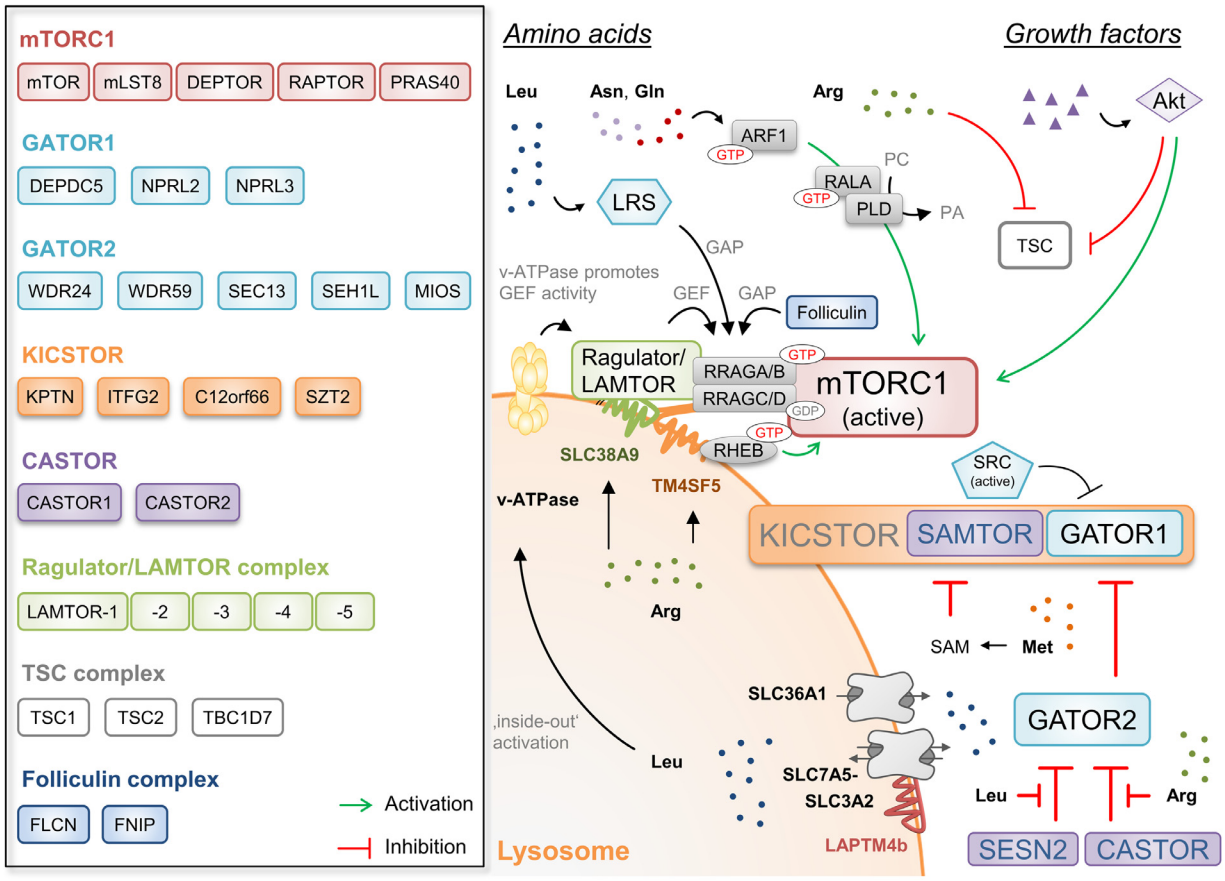

Fig. 3. Activation of mTORC1 by amino acids and growth factors leads to inhibition of autophagy. Upon stimulation, mTORC1 activation by $RHEB^{GTP}$ occurs at the lysosomal membrane (RRAG GTPase-dependent). TSC2-mediated repression of RHEB is eliminated by TSC sequestration to the cytosol by arginine and AKT. AKT-mediated binding of 14-3-3 to PRAS40 prevents mTORC1 auto-inhibition. Cytosolic and lysosomal amino acid availability modulates RRAG GTPase nucleotide loading states, requiring GAP activity of FLCN-FNIP and LRS as well as GEF activity of the Ragulator/LAMTOR complex, which is stimulated by v-ATPase and arginine-dependent SLC38A9 interaction. Intra-lysosomal amino acids, mainly leucine and arginine, are sensed by v-ATPase and SLC38A9 respectively, which boost transport of amino acids. Furthermore, high lysosomal arginine levels promote interaction between TM4SF5, SLC38A9 and mTORC1 for mTORC1 activation. High lysosomal leucine levels are mediated by SLC7A5/SLC3A2 (interacted with LAPTM4B), promoting v-ATPase assembly to promote GEF activity of the Ragulator/LAMTOR complex (inside-out-activation). Cytosolic leucine and arginine indirectly modulate RRAG GTPases via SESN2- or CASTOR-GATOR2-GATOR1-axis. Cytosolic methionine-dependent mTORC1 activation involves the cytosolic amino acid sensors SAMTOR supercomplexed with KICSTOR and GATOR1. SRC promotes the dissociation of GATOR1 from RRAG GTPases. Cytosolic asparagine and glutamine signal to mTORC1 through ARF1, involving small GTPase RALA and PLD. PA, produced by PLD, delivers mTORC1 to the lysosome for activation.

Abbreviations: AKT, AKT Serine/Threonine Kinase; ARF1, ADP Ribosylation Factor 1; Arg, arginine, Asn, asparagine; C12orf66, Chromosome 12 Open Reading Frame 66; CASTOR, Cytosolic Arginine Sensor For mTORC1; DEPDC5, DEP Domain Containing 5; DEPTOR, DEP Domain-Containing mTOR-Interacting Protein; FLCN, Folliculin; FNIP, Folliculin Interacting Protein; GAP, GTPase-Activating Proteins; GATOR, GAP activity towards RAG; GDP, Guanosine diphosphate; GEF, Guanosine Triphosphate Exchange Factor; Gln, glutamine; GTP, guanosine triphosphate; ITFG2, Integrin Alpha FG-GAP Repeat Containing 2; KICSTOR, KPTN, ITFG2, C12orf66, and SZT2-containing regulator of mTORC1; KPTN, Kaptin, Actin Binding Protein; LAMTOR, Late Endosomal/Lysosomal Adaptor And MAPK And MTOR Activator; LAPTM4B, Lysosomal Protein Transmembrane 4 Beta; Leu, leucine; LRS, Leucyl-tRNA Synthetase; Met, methionine; MIOS, Meiosis Regulator For Oocyte Development (Homolog (Drosophila)); mLST8, Mammalian Lethal with SEC13 Protein 8; mTOR, mechanistic Target Of Rapamycin; NPRL2/3, Nitrogen Permease Regulator-Like Protein; PA, phosphatidic acid; PC, phosphatidylcholine; PLD, Phospholipase D; PRAS40, Proline-Rich AKT Substrate Of 40 kDa; RALA, RAS Like Proto-Oncogene A; RAPTOR, Regulatory-Associated Protein Of mTOR; RHEB, RAS Homolog Enriched In Brain; RRAGA/B/C/D, RAS Related GTP Binding A/B/C/D; SAM, S-adenosylmethionine; SAMTOR, S-Adenosylmethionine Sensor Upstream Of mTORC1; SEC13, SEC13 Homolog, Nuclear Pore And COPII Coat Complex Component; SEH1L, SEH1 Like Nucleoporin; SESN2, Sestrin-2; SLC, Solute Carrier; SRC, Proto-Oncogene Tyrosine-Protein Kinase SRC (short for sarcoma); SZT2, Seizure Threshold 2 Protein Homolog; TBC1D7, TRE2-BUB2-CDC16 (TBC) 1 Domain Family Member 7; TM4SF5, Transmembrane 4 Superfamily Member 5; TSC1/2, Tuberous Sclerosis Complex Protein 1/2; WDR24/59, WD Repeat Domain 24/59.

affinity tethering of DEPDC5 to RRAGA sterically prevents access of NPRL2/3 to RRAGA/C and is therefore characterized by a low GAP activity ('inhibitory mode'), whereas a high GAP activity is induced by a weak affinity binding of NPRL2/3 to RRAGA/C ('GAP mode') [81,82]. In addition to its GAP function under amino acid deficiency, NPRL2 was surprisingly shown to exhibit a positive function on mTORC1 through interaction with RAPTOR during amino acid sufficiency [85].

Inhibition of mTORC1 via the GAP function of GATOR1 in response to nutrient starvation is highly conserved in evolution, with amino acid depletion in mammals and nitrogen-sources in yeast being best described for the induction of macroautophagy. It has also been reported that the GATOR1 complex in yeast directly induces macroautophagy, e.g. by dephosphorylation of Atg13p, and mitophagy by altering the carbon source (glucose-lactose switch) [79], which can be reversed by adding a single amino acid methionine [83] (see also methionine regulation below).

During the search for further interaction partners to enhance the understanding of GATOR1's function on RRAG GTPases, the tetrameric complex KICSTOR was identified. This complex is named for Kaptin (KPTN), Integrin Alpha FG-GAP Repeat Containing 2 (ITFG2), C12orf66, and Seizure Threshold 2 (SZT2)-Containing Regulator Of mTORC1 [84], wherein SZT2 serves as a linker to form the tetrameric complex. KICSTOR, which is localized to lysosomes, has been shown to bind and recruit GATOR1 through the interaction between SZT2 and DEPDC5. This interaction was found to be necessary for the interaction between GATOR1 and RRAG GTPases, as well as for the mTORC1 inhibitory effect of GATOR1 [84]. Furthermore, the loss of SZT2 led to the disruption of the GATOR1–GATOR2 interaction, suggesting an additional role of KICSTOR to enable GATOR2 inhibitory function to GATOR1 [84,85]

Furthermore, in the absence of amino acids, the GDP-bound state of RRAGA/B leads to the recruitment of the Folliculin (FLCN)–Folliculin-Interacting Protein (FNIP) complex (FLCN-FNIP) to the lysosomes. FLCN-FNIP is a potent GAP for RRAGC/D and is thought to play an important role in lysosomal recruitment of mTORC1 by returning RRAG GTPases back to their active conformation after amino acid repletion [69,86]. During starvation, lysosomal localization of FLCN-FNIP requires GATOR1 to place FLCN under the control of the amino acids sensors upstream of GATOR1 [87]. Not much is known about the regulation of FLCN-FNIP, but when amino acids become available, the FLCN-FNIP is directly in place to convert RRAGC/D into the GDP-bound active form, promoting mTORC1 activation and macroautophagy inhibition (Fig. 2).

Besides nutrient availability, macroautophagy is also regulated by energy availability. Low energy conditions are reflected by an increased AMP:ATP ratio, which activates the AMP-Activated Protein Kinase (AMPK) [88,89]. In this context, AMPK can regulate macroautophagy directly via mTORC1, ULK1 and the PI3KC3-C1 complex and indirectly via the expression of autophagy-related genes downstream of various transcription factors, such TFEB [90]. The inhibition of mTORC1 is achieved by phosphorylation of RAPTOR at two highly-conserved serine residues (S722/792), which induces 14-3-3 protein binding to RAPTOR and subsequently suppresses mTORC1 kinase activity [91]. The 14-3-3 protein binding to phosphorylated mTORC1 members plays a crucial role in mTORC1 activity. When growth factors are present, Serine/Threonine Protein Kinase B (PKB), also known as AKT, phosphorylates mTORC1-auto-inhibitory PRAS40 and opposes its activity through 14-3-3 binding, leading to mTORC1 activation [91]. Both AMPK and AKT phosphorylate different sites of TSC to activate or inhibit TSC2 GAP activity, respectively, and thus control additionally RHEB-associated mTORC1 activity [92–94].

The reactivation of mTORC1, and thus the inactivation of macroautophagy, can be achieved if the cell has sufficient nutrients (e.g. amino acids) available either by the lysosomal degradation of macromolecules or by the provision of nutrients by external sources. For this process, the RRAG GTPases must reassume their active conformation – a process which requires (i) GAP activity of FLCN-FNIP towards RRAGC/D [69], (ii) inhibition of GAP activity of GATOR1 towards RRAGA/B as well as (iii) GEF activity of the Ragulator/LAMTOR complex towards RRAGA/B [73,74] (Fig. 2B).

As mentioned earlier, it is known that the GATOR2 complex inhibits GATOR1 in the presence of amino acids [5,74], and that within GATOR1, NPRL3 is necessary for the interaction between GATOR1 and GATOR2 [80]. Compared to GATOR1, GATOR2 consists of five proteins, namely WD Repeat-Containing Proteins 24 and 59 (WDR24, WDR59), Meiosis Regulator For Oocyte Development (MIOS), SEC13 Homolog, Nuclear Pore And COPII Coat Complex Component (SEC13), and SEH1 Like Nucleoporin (SEH1L) [74]. However, its structure and molecular function still needs to be elucidated. One possible explanation for the activity of GATOR1 being suppressed by GATOR2 has been described by the function of the tyrosine kinase SRC, which has been shown to promote the dissociation of GATOR1 from RRAG GTPases in the presence of amino acids. However, how the amino acid signal is transferred to SRC is not clear, but the authors suggest that GATOR2 may be a possible candidate [95]. Considering the individual GATOR2 complex proteins, it has been shown that the depletion of almost all GATOR2 proteins (WDR59, WDR24, SEH1L, MIOS) leads to a

decrease in mTORC1 activity and abolishes restimulation with amino acids [74,96,97], indicating that the integrity of the GATOR2 complex is essential for the GATOR2-dependent inhibition of GATOR1. Interestingly, WDR24 was identified to play an mTORC1-independent role in the regulation of lysosome dynamics, especially lysosomal acidification, as well as autophagic flux *in vivo* in *Drosophila* and human HeLa cells. Similar observations were made for MIOS and SEH1L [96]. In addition, WDR24 depletion induces defective embryogenesis in zebrafish, which could also be partially explained by a dysregulated autophagy [98]. These observations suggest a non-canonical function for GATOR2 in macroautophagy regulation, mainly by impacting autophagic flux and the fusion of the autophagosome with the lysosome. Furthermore, WDR59 and MIOS have been identified as interactors of the E3 ubiquitin ligase HERC2, and WDR59 as DDB1 and CUL4 Associated Factor (DCAF) of the Cullin4-DNA Damage Binding Protein 1 (CUL4-DDB1) ubiquitin ligase complex, which among other things play an important role in DNA repair and genomic integrity [99,100]. These interactions may be indicative of another role for GATOR2 in the regulation of DNA repair mechanisms in addition to its role in the regulation of GATOR1 and mTORC1-independent regulation of macroautophagy.

In the latter case, the GEF activity of the Ragulator/LAMTOR complex correlates with the interaction between the Ragulator/LAMTOR complex and the v-ATPase and its ability to hydrolyze ATP (Fig. 2B). The best-described function of the v-ATPase is the transport of protons (H^+) and thus the acidification of the lysosomal lumen. However, it was shown that ATP hydrolysis and the assembly of v-ATPase subunits and not the function of lysosomal acidification by v-ATPase is necessary for the regulation of mTORC1 signaling [101]. Under amino acid deprived conditions the interaction between the Ragulator/LAMTOR complex and v-ATPase is strengthened and blocks GEF activity. After restimulation, the interaction becomes weaker, by induction of a conformational change between v-ATPase and the Ragulator/LAMTOR complex, more precisely LAMTOR4 (HBXIP) and LAMTOR5 (C7orf59), which stimulates GEF activity [73,101]. This v-ATPase-Ragulator/LAMTOR axis to activate mTORC1 in response to certain amino acids functions either upstream or in parallel to GATOR1-mediated mTORC1 inhibition, since in GATOR1-null cell lines, where mTORC1 is hyperactive and non-responsive to amino acid regulation, pharmacological inhibition of the v-ATPase does not reduce mTORC1 activity [74].

Hereinafter, immunoprecipitation of Ragulator/LAMTOR components led to the identification of Sodium-Coupled Neutral Amino Acid Transporter 9 (SLC38A9; SLC38A9.1). Similar to the GEF-activation mechanism of the Ragulator/LAMTOR complex by the v-ATPase-Ragulator/LAMTOR interaction described above, the interaction of SLC38A9 with the Ragulator/LAMTOR complex and RRAG is enhanced under amino acid-deficient conditions [102]. Both, SLC38A9 and v-ATPase represent amino acid dependent pathways that converge on the Ragulator/LAMTOR-RRAG complex.

In summary, mTORC1 acts as a central regulator of cell homeostasis, whereby under favorable conditions the activity of mTORC1 directly mediates cell growth while suppressing macroautophagy. Thereby, the activity of mTORC1 is influenced by a variety of extrinsic and intrinsic factors, with the regulation of the nucleotide loading state of the GTPases RHEB and RRAG (Fig. 2) as well as the lysosomal recruitment of mTORC1 being of essential importance.

Regulation of GTPases, mTORC1 and macroautophagy by amino acid sensory proteins

The ability to perceive changes in the intracellular abundance of amino acids and to respond to them by terminal regulation of mTORC1 activity, and thus macroautophagy, is mediated by a complex network of amino acid sensors. Especially in the last decade, various proteins and protein complexes with different subcellular localization have been characterized. In this context, intra-lysosomal and cytoplasmic amino acid sensing is best described (Fig. 3).

Intra-lysosomal sensing

Lysosomes are the endpoint of many catabolic pathways, such as macroautophagy, and thus serve as a provider of amino acids for reactivation of mTORC1 in the event of amino acid deficiency and concomitant mTORC1 inactivation [41,103]. Furthermore, besides their function in intracellular amino acid storage and signaling, lysosomes are able to integrate intra- and extracellular signals through secretory, endocytic, autophagic and phagocytic trafficking pathways [104–106]. As mentioned in the previous section, v-ATPase plays an essential role in the recruitment and activation of mTORC1 on the lysosomal surface. Some studies suggest that v-ATPase itself acts as an amino acid sensor [73,101,107] and that the assembly of the v-ATPase subdomains, ATP-hydrolytic V_1 domain and the proton-conducting V_0 domain important for v-ATPase activity, may play an important role in this process [108–110]. The stimulation of v-ATPase by intra-lysosomal amino acids and the subsequent activation of mTORC1 by the interplay of Ragulator/

LAMTOR, RRAG GTPases and RHEB is described as an inside-out activation mechanism.

Especially the sensing and regulation of the intra-lysosomal leucine concentration was investigated in this regard. The uptake of the essential amino acid leucine into the lysosomal lumen is facilitated by the Lysosomal Protein Transmembrane 4b (LAPTM4b)-dependent recruitment of the heterodimeric leucine transporter LAT1-4F2HC (SLC7A5-SLC3A2) to lysosomes [111]. The resulting high intra-lysosomal leucine concentrations promote ATP hydrolysis and assembly of the v-ATPase to support mTORC1 recruitment to lysosomes [101,109,111]. Another mechanism of controlling the amount of leucine within lysosomes is the interaction and reciprocal regulation of FLCN and the lysosomal transporter SLC36A1 (PAT1 or LYAAT-1). Similar to the accumulation of SLC36A1 in the lysosomal membrane upon amino acid deprivation, the depletion of FLCN leads also to an increased accumulation of SLC36A1 and thus to an increased efflux of leucine and subsequent inhibition of mTORC1. This effect could be reversed by the overexpression of FLCN, resulting in mTORC1 stimulation, albeit amino acids are deprived [112]. In addition, as a proton-coupled amino acid transporter, SLC36A1 was also shown to be sensitive to v-ATPase inhibition, which led to the accumulation of lysosomal metabolites. Interestingly, the majority of essential amino acids, such as leucine, was not affected by v-ATPase-inhibition, most likely due to a still ongoing efflux. Instead, nutrient starvation was necessary to prevent the egress of essential amino acids from lysosomes, which was ascribed to GATOR1-dependent mTORC1 regulation [113]. In summary, the lysosomes can be described as sensing and dynamic storage organelles for amino acids, whereby the lysosomal concentrations of essential amino acids, such as leucine, are regulated by an mTOR-dependent mechanism and those of non-essential amino acids by v-ATPase-dependent mechanisms [113].

Besides leucine, the intra-lysosomal sensing of the conditionally essential amino acid arginine was also studied. At the lysosomal membrane, the Ragulator/LAMTOR complex was shown to interact with v-ATPase and additionally with the amino acid transporter SLC38A9 [102], a lysosomal effluxer of many essential non-polar amino acids, such as leucine [103] (Fig. 1 and 3). SLC38A9 has been shown to be an integral and probably a stoichiometric member of the Ragulator/LAMTOR-RRAG complex, sensing amino acid levels and controlling mTORC1 [114]. Mechanistically, lysosomal arginine binds to SLC38A9 that in turn undergoes conformational changes to stimulate GTP-loading of RRAGA GTPase. Thus, SLC38A9 and Ragulator/LAMTOR both function as GEFs that trigger mTORC1 activation in response to nutrient sufficiency [82,102,103,114] (see also Fig. 2). In addition, based on the literature [103,115,116] it can be assumed that an increase in cytoplasmic leucine levels mediated by efflux via SLC38A9 can be detected by mTORC1 through cytosolic Sestrin (SESN) protein family proteins, allowing lysosomes to cross-talk with cytosolic amino acid sensors (see cytosolic sensing). In a recent study, an additional transmembrane protein (Transmembrane 4 Super Family Member (TM4SF5)) was identified to be a potential lysosomal arginine sensor [117]. Under physiological arginine levels, TM4SF5 interacts with SLC38A9 and mTORC1, possibly promoting arginine efflux through SLC38A9 by sensing and signaling lysosomal arginine levels to SLC38A9 for mTORC1 activation.

Cytosolic sensing

As already mentioned, cell metabolism and macroautophagy are fine-tuned by the availability of amino acids, which must therefore also be perceived in the cytosol. Similar to the intra-lysosomal amino acid sensing, the majority of studies in cytosolic amino acid sensing were also focused on leucine. The first identified leucine sensor acting on mTORC1 is the tRNA charging enzyme Leucyl tRNA-Synthetase (LRS), which was shown to interact with RRAGD [118,119]. More specifically, in the presence of amino acids, LRS translocates to lysosomes, binds leucine-dependently to $RRAGD^{GTP}$ and functions as a GAP for RRAGD [119], thus promoting mTORC1 activation [68]. In addition, the knock-down of LRS prevents the translocation of mTORC1 to the lysosomal surface and activates macroautophagy, detectable by an increase in the number of GFP-LC3-II puncta [119]. These results indicate that LRS is an important regulator of the mTORC1-macroautophagy regulatory circuit.

A crucial regulator acting in parallel to LRS in cytosolic leucine sensing was described with the discovery of the SESN-GATOR axis, in which GATOR1/2 complexes are of central operational importance [76,120,121]. The highly conserved and stress-induced proteins Sestrin 1, 2 and 3 (SESN1, SESN2, SESN3) were reported to function as putative cytosolic amino acid sensors for leucine. However, most studies are currently focused on SESN2 [7,76,115,116,121,122]. It should also be noted that the leucine sensing function of SESNs has been questioned and they are rather described as generally stress-induced proteins that contribute to the maintenance of cellular homeostasis by linking metabolism and energy balance [123]. Independent of this controversy, the functioning of the SESN proteins was described as follows. During leucine starvation, SESNs directly interact with the GATOR2 members WDR24 and SEH1L impeding GATOR1 inhibition by GATOR2 [76]. In contrast, leucine-bound SESN2 is unable to block GATOR2, which

consequently activates mTORC1 by impeding GAP activity of GATOR1 (Fig. 3). SESN2, leucine and GATOR2 exist in a defined equilibrium. In short-term leucine deprivation, leucine-free SESN2 binds GATOR2, permitting the GAP activity of GATOR1 towards RRAGA/B, promoting GDP-binding and inhibiting mTORC1 pathway. Under conditions of cellular stress, including long-term amino acid deprivation, SESN2 levels were shown to be increased and subsequently extend SESN2-mediated inhibition of GATOR2. As a result of the change in the equilibrium, more leucine is required to achieve the same degree of mTORC1 activity, which consequently renders the mTORC1 complex less sensitive to leucine fluctuations [7]. In addition to influencing mTORC1 activity by expression, phosphorylation of SESN2 has been shown to play a role in the transmission of the leucine signal to GATOR2. Here, leucine deficiency induces ULK1-dependent phosphorylation of SESN2 at T232, S249, and S279, which leads to an enhanced interaction of SESN2 and GATOR2 and ultimately to an increased inhibition of mTORC1 [124]. Furthermore, SESN2 was also reported to function as a guanine nucleotide dissociation inhibitor (GDI) for RRAGA/B counteracting GEF activity to keep RRAGA/B in their inactive conformation [125]. However, in a recent structural analysis, SESN2 shows no similarity to known GDI proteins and the putative GDI peptide motif is not present on the protein surface in SESN2 [115]. Thus, leucine-dependent indirect GAP activity to RRAGA/B through the SESN2-GATOR2-GATOR1 axis is the more likely function of SESN2 in the RRAG-GTPase cycle [115] (see also Fig. 2).

In addition to their function in the leucine-dependent regulation of the RRAG GTPase cycle, a large number of studies indicate that SESNs positively influence the induction of macroautophagy in response to various environmental stresses [126]. With respect to SESNs antioxidant function, SESN2 was shown to bind to the selective autophagy receptor SQSTM1 to promote the autophagic degradation of Kelch-Like ECH Associated Protein 1 (KEAP1). This leads to an increased stability of the antioxidant transcription factor Nuclear Factor Erythroid 2 Like 2 (NRF2) and thus to an induction of antioxidant genes [127]. The study by Ro *et al*. showed that SESN2 forms a complex with ULK1 and SQSTM1/p62, in which SESN2 promotes the phosphorylation of SQSTM1/p62 on S403 by ULK1 under energetic stress [128].

Furthermore, SESN2 was found to be involved in the regulation of mitochondrial processes, such as oxidative phosphorylation and the selective macroautophagy process of mitophagy [129–132]. Again, ULK1 plays a central role in these processes. In response to copper-induced oxidative stress, SESN2 is phosphorylated by ULK1 at S73 and S254 and associates with the mitochondrial protein ATP Synthase F1 Complex Alpha Subunit 1 (ATP5A) on damaged mitochondria. Furthermore, in macrophages SESN2 plays a dual role in inducing mitophagy in response to inflammasome activation. On the one hand, SESN2 induces the recognition mechanism of defective mitochondria by mediating the aggregation of SQSTM1/p62 and its binding to K63-ubiquitinated mitochondria. At the same time, SESN2 activates mitophagy by increasing ULK1 protein levels [129]. In summary, these studies indicate that the SESN2-ULK1 interplay plays an important part in regulating macroautophagy induction and mitophagy in response to various cellular stress factors, including leucine deficiency.

As already mentioned in the previous section, another critical amino acid for mTORC1 signaling is arginine. Intra-lysosomal arginine is sensed by SLC38A9 [102]. However, cells lacking SLC38A9 were shown to be still sensitive to arginine starvation due to the function of the Cytosolic Arginine Sensor for mTORC1 (CASTOR1). CASTOR1 can either form a homodimer or heterodimerizes with CASTOR2. Similar to the SESN function in leucine sensing, the CASTOR complex interacts with and inhibits GATOR2 releasing the GAP activity of GATOR1 to inhibit mTORC1 during arginine deprivation. However, binding of arginine to CASTOR1, but not to CASTOR2, leads to inhibition of the CASTOR complex. Thus, only when cells lack CASTOR1 and SLC38A9 they are fully insensitive to arginine starvation [133].

Structural analysis of arginine-bound CASTOR1 showed that each monomer of the CASTOR1 homodimer has four tandem ACT domains (Aspartate kinase, Chorismate mutase, TyrA) (ACT1-4), of which the interface of ACT2 and ACT4 forms a pocket for arginine binding [134]. CASTOR2 shares more than 60% sequence identity with CASTOR1, but does not bind arginine due to differences in two amino acid residues in this ACT2-ACT4 interface. Nevertheless, CASTOR2 is still able to bind GATOR2 [134,135]. For CASTOR1, opposing the arginine-binding pocket, five highly conserved residues are required for GATOR2 interaction, most notably the aspartate residue D121, which is not present on the surface of the protein domain when arginine is bound. This effect by arginine binding was suggested as a possible explanation for how arginine prevents CASTOR1-GATOR2 interaction [134]. The study by Zhou *et al.* showed that the arginine binding to CASTOR1 serves as a linker between the ACT2 and ACT4 domains and does not induce a conformational change in CASTOR1, since CASTOR1 possesses similar structures in the apo- and arginine-bound states [136]. The authors describe the linking between ACT2 and ACT4 by arginine as an inhibition of an unidentified

conformational change that is essential for the stable CASTOR1-GATOR2 interaction. However, CASTOR1 D121 mutants, which are unable to bind GATOR2, failed to inhibit mTORC1 signaling by impaired GATOR1 inhibition [134]. The binding of GATOR2 to CASTOR1 is mediated by MIOS, whereby the binding affinity was described as weak [137]. According to this, the weak interaction between CASTOR1 and GATOR2 (MIOS) and the fact that the structure of CASTOR1 is similar in the apo- and arginine-bound state suggests unidentified binding partners involved in the interaction between GATOR2 and CASTOR1. However, further investigations are necessary.

In addition to describing the canonical function of the SESN/CASTOR-GATOR1/2-RRAG axis in response to extracellular-derived amino acids, the recent publication by Hesketh *et al*. has revealed that this amino acid sensing axis can also have a negative regulatory function on the activation of mTORC1 after stimulation by lysosomal proteolysis-derived amino acids. In addition, the authors could show that this stimulation of mTORC1 is dependent on the integrity of the HOPS complex and thus also describes a previously unknown function of this complex [138]. Although the underlying mechanism remains to be elucidated in the future, this finding highlights the close interplay between lysosomal amino acid sensing mechanisms and the function of cytoplasmic mTORC1 regulatory complexes and amino acid sensors.

Together with leucine, arginine represents the main contributor to amino acid-dependent mTORC1 activity. Besides the cytoplasmic arginine-sensing mechanism, involving CASTOR-GATOR1/2-RRAG, arginine has also been shown to have a parallel effect on mTORC1 independently of GATOR1/2/RRAG via the TSC2-RHEB signaling [139]. It is well established that under growth factor deprivation TSC2 negatively regulates mTORC1, but the removal of both serum and arginine was required to fully inhibit mTORC1, suggesting an additional role for arginine to support low levels of mTORC1 activity under serum-deprived conditions. Arginine has been shown to cooperate with growth factors by interfering with the TSC2-RHEB interaction and inhibiting the recruitment of TSC2 to lysosomes, thus allowing maximal mTORC1 activation when growth factors become available [139].

As depicted in Fig. 3, an additional amino acid that is sensed in the cytosol is the essential amino acid methionine. In addition to its important role in protein structures and as the initiating amino acid in eukaryotic protein synthesis, this amino acid has a variety of metabolically unique features. In particular, the presence of sulfur enables a redox cycle with reversible oxidation and reduction, which in cellular anti-oxidative defense and as a translational modification influences redox sensing and regulation [140,141]. Regarding its role in mTORC1 regulation, methionine is not directly sensed, but the methionine-derived metabolite S-adenosylmethionine (SAM), which mainly contributes as a methyl-donating substrate to various cytosolic or nuclear processes, e.g. methylation of DNA and one-carbon-metabolism [142]. In mammalian cells, the S-Adenosylmethionine Sensor Upstream Of mTORC1 (SAMTOR) was identified as the sensor of SAM, which was additionally described as a binding protein of GATOR1, more precisely DEPDC5. Thereby, SAMTOR was only co-immunoprecipitated when KICSTOR was present. However, SAMTOR has been shown to interact to all known GATOR1 and KICSTOR components and both complexes are required for the interaction. In absence of methionine/SAM, SAMTOR binds to GATOR1-KICSTOR to positively modulate GATOR1's GAP activity, thus acts as a negative regulator of mTORC1 [143]. In contrast, in the presence of methionine, SAM disrupts the interaction between SAMTOR and the GATOR1-KICSTOR supercomplex, and thus promotes mTORC1 activation. Additionally, SAMTOR was also shown to bind S-adenosylhomocysteine (SAH), the demethylated form of SAM, resulting in the same effect on mTORC1 [143]. Taken together, unlike SESN and CASTOR, which oppose GATOR2 function, SAMTOR links purine metabolism and redox balance to mTORC1-regulated processes through GATOR1.

In yeast, where no SAMTOR homologue is found, methionine can still be sensed through the regulated methylation of the phosphatase 2A (PP2A) family [83,144]. High intracellular SAM levels are accompanied by increased methylations, resulting in the activation of PP2A by the production of methyl-PP2A. In yeast, methyl-PP2A dephosphorylates and inactivates Npr2 (yeast homologue of mammalian NPRL2), which in turn leads to increased TORC1 activity. However, no report has shown whether methyl-PP2A is directly involved in the inactivation of mammalian NPRL2. For further details of mTORC1-activating mechanisms by methionine see [145].

Although amino acids like leucine, arginine or methionine require RAG GTPases for lysosomal localization and subsequent activation of mTORC1, glutamine and asparagine do not [71,146]. Glutamine and asparagine signal to mTORC1 through the small GTPase ARF1, a pathway that is independent on RAG GTPases. This ARF1-dependent mechanism requires the small GTPases RALA and RHEB as well as PLD. PLD catalyzes the production of phosphatic acid (PA), which has been described to be required for the stability and activation of mTORC1 [147,148]. Furthermore, exogenously supplied vesicles containing PA have been shown to deliver mTORC1 to lysosomes in the absence of amino acids, growth factors, RRAGC/D

and RHEB GTPases [149]. Like ARF1, both RALA and PLD are known to be involved in vesicle trafficking processes [150], suggesting that for RRAG-independent mechanisms, this feature facilitates lysosomal localization of mTORC1 to activate mTORC1 by RHEB (Fig. 3).

Amino acids sensing at the Golgi apparatus

The discovery of RRAG-independent sensing mechanisms and the fact that RHEB can also be located at the Golgi apparatus, strongly suggests that a metabolic sensing machinery is also present at this organelle. Indeed, the small GTPase RAB1A has been shown to transmit the amino acid signal to activate mTORC1 independently of RRAGs and lysosomes [151]. In a further study, the Golgi-localized RAB1A and the amino acid transporter SLC36A4 (PAT4) were identified as the amino acid sensing machinery that interacts with RAPTOR and mTOR to recruit mTORC1 in proximity to RHEB [152] (Fig. 3).

However, to shed light on how mTORC1 is activated by amino acids on cell organelles other than lysosomes, a number of further investigations are necessary. With the identification of additional small GTPases, RRAGs are not the only metabolic relays to regulate mTORC1. And thus, it is very likely that they are replaced by other small GTPases on other organelles.

Conclusions

Macroautophagy is an indispensable metabolic process for cellular homeostasis, which is finely regulated by the presence or absence of amino acids. Thereby, the consensus of the amino acid sensing mechanism described here is the stimulation of small GTPases, especially the RRAG-GTPases, which act as metabolic relays to convey amino acid availability to the central macroautophagy-regulator mTORC1.

The complexity of these processes is reflected in the multitude of proteins and protein complexes, which serve as intra-lysosomal and cytosolic sensors of amino acids, especially leucine, arginine and methionine. Structural analysis in particular has made a major contribution in recent years to better understand the exact functioning of these sensor proteins and sensor complexes. And although the cytosolic and intra-lysosomal amino acid sensing is quite well-defined, it remains to be seen which proteins and metabolites control the sensing processes in other organelles to maintain cellular homeostasis. Furthermore, it remains to be elucidated to what extent mTORC1-independent functions of sensory proteins, e.g. the mTORC1-independent regulation of macroautophagy by GATOR2 proteins, will add another level of complexity.

Author contributions

All authors took part in developing the outline, drafting sections, and editing the entire manuscript. All authors have reviewed and approved the article. None of the authors have any potential conflicts of interest to disclose

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Abbreviations:

ACT, Aspartate Kinase, Chorismate Mutase, TyrA; AKT, AKT Serine/Threonine Kinase; ALR, Autophagic Lysosome Reformation; AMBRA, Beclin 1-Regulated Autophagy Protein 1; AMP/ATP, adenosine mono-/triphosphate; AMPK, AMP Activated Kinase; ARF1, ADP Ribosylation Factor 1; Arg, arginine; ARL8b, ADP Ribosylation Factor Like GTPase 8B; Asn, asparagine; ATG, Autophagy Related Gene; BECN1, Beclin-1; C12orf66, Chromosome 12 Open Reading Frame 66; C7orf59, Chromosome 7 Open Reading Frame 59; CASTOR, Cytosolic Arginine Sensor for mTORC1; CHMP2A, Charged Multivesicular Body Protein 2A; CLEAR, Coordinated Lysosomal Expression And Regulation; CMA, Chaperone-Mediated Autophagy; CUL4, Cullin 4; DCAF, DDB1 And CUL4 Associated Factor; DDB1, DNA Damage Binding Protein 1; DEPDC5, DEP Domain Containing 5; DEPTOR, DEP Domain-Containing mTOR-Interacting Protein; DFCP1, Double FYVE Domain-Containing Protein 1; E1, Ubiquitin-Activating Enzyme; E2, Ubiquitin-Conjugating Enzyme; E3, Ubiquitin Ligase; ER, Endoplasmic Reticulum; ESCRT, Endosomal Sorting Complexes Required for Transport; FIP200, Focal Adhesion Kinase (FAK)-Family Interacting Protein 200; FLCN, Folliculin; FNIP, Folliculin Interacting Protein; GABARAP, γ-Aminobutyric Acid Receptor-Associated Protein; GABARAPL1/2, GABARAP-like 1/2; GAP, GTPase-Activating Proteins; GATOR, GAP Activity Towards RRAG; GDI, Guanine Nucleotide Dissociation Inhibitor; GDP, guanosine

diphosphate; GEF, Guanosine Triphosphate Exchange Factor; GFP, Green Fluorescent Protein; Gln, glutamine; GSK3b, Glycogen Synthase Kinase 3 Beta; GTP, guanosine triphosphate; HBXIP, Hepatitis B Virus X-Interacting Protein; HERC2, HECT And RLD Domain Containing E3 Ubiquitin Protein Ligase 2; HOPS, Homotypic Fusion And Protein Sorting; HSC, Heat Shock Cognate Protein Of 70 kDa; HUWE1, HECT, UBA And WWE Domain Containing E3 Ubiquitin Protein Ligase 1; ITFG2, Integrin Alpha FG-GAP Repeat Containing 2; KEAP1, Kelch-Like ECH Associated Protein 1; KICSTOR, KPTN, ITFG2, C12orf66, And SZT2-Containing Regulator Of mTORC1; KPTN, Kaptin, Actin Binding Protein; LAMTOR, Late Endosomal/Lysosomal Adaptor And MAPK And mTOR Activator; LAPTM4B, Lysosomal Protein Transmembrane 4 Beta; LC3, Light Chain 3; Leu, leucine; LRS, Leucyl-tRNA Synthetase; MAP1LC3B, Microtubule Associated Protein 1 Light Chain 3 Beta; Met, methionine; MIOS, Meiosis Regulator For Oocyte Development (Homolog (Drosophila)); MiT/TFE, Microphthalmia/Transcription Factor E; mLST8, Mammalian Lethal with SEC13 Protein 8; MP1, Mitogen-Activated Protein Kinase Kinase 1-Interacting Protein 1; mTOR, mechanistic Target Of Rapamycin; mTORC1, Mechanistic Target Of Rapamycin Complex 1; NPRL2/3, Nitrogen Permease Regulator-Like Protein; NRBF2, Nuclear Receptor-Binding Factor 2; NRF2, Nuclear Factor Erythroid 2 Like 2; PA, phosphatidic acid; PACER, Protein Associated With UVRAG As Autophagy Enhancer; PAS, Phagophore Assembly Site; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI(3)P, phosphatidylinositol (3)-phosphate; PI3KC3-C1/2, Class III Phosphatidylinositol 3-Kinase Complex 1/2; PKB, Serine/Threonine Protein Kinase B; PLD, Phospholipase D; PRAS40, Proline-Rich AKT Substrate Of 40 kDa; RAB7, Ras-Related Protein RAB7; RALA, RAS Like Proto-Oncogene A; RAPTOR, Regulatory-Associated Protein Of mTOR; RHEB, Ras Homolog Enriched In Brain; RRAGA/B/C/D, Ras Related GTP Binding A/ B/ C/ D; RUBICON, Run Domain Protein as Beclin-1 Interacting And Cysteine-Rich Containing; SAM, S-Adenosylmethionine; SAMTOR, S-Adenosylmethionine Sensor Upstream of mTORC1; SEC13, SEC13 Homolog, Nuclear Pore And COPII Coat Complex Component; SEH1L, SEH1 Like Nucleoporin; SESN2, Sestrin-2; SLC, Solute Carrier; SQSTM1, Sequestosome 1; SRC, Proto-Oncogene Tyrosine-Protein Kinase Src (short for sarcoma); STX-17, Syntaxin-17; SZT2, Seizure Threshold 2 Protein Homolog; TBC1D7, Tre2-Bub2-Cdc16 (TBC) 1 Domain Family Member 7; TFEB, Transcription Factor EB; TM4SF5, Transmembrane 4 Superfamily Member 5; TSC1/2, Tuberous Sclerosis Complex Protein 1/2; ULK1/2, Unc-51 Like Autophagy Activating Kinase 1/2; UVRAG, Ultraviolet Radiation Resistance-Associated Gene Protein; VPS, Vacuolar Protein Sorting-Associated Protein; WDR24/59, WD Repeat Domain 24/59; WIPI2, WD Repeat Domain Phosphoinositide-Interacting Protein 2; XPO1, Exportin 1

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