J. Indian Inst. Sci.
A Multidisciplinary Reviews Journal
ISSN: 0970-4140 Coden-JIISAD

REVIEW ARTICLE

Conceptualizing Eukaryotic Metabolic Sensing and Signaling

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Abstract | For almost all cells, nutrient availability, from glucose to amino acids, dictates their growth or developmental programs. This nutrient availability is closely coupled to the overall intracellular metabolic state of the cell. Therefore, cells have evolved diverse, robust and versatile modules to sense intracellular metabolic states, activate signaling outputs and regulate outcomes to these states. Yet, signaling and metabolism have been viewed as important but separate. This short review attempts to position aspects of intracellular signaling from a metabolic perspective, highlighting how conserved, core principles of metabolic sensing and signaling can emerge from an understanding of metabolic regulation. I briefly explain the nature of metabolic sensors, using the example of the AMP activated protein kinase (AMPK) as an "energy sensing" hub. Subsequently, I explore how specific central metabolites, particularly acetyl-CoA, but also *S*-adenosyl methionine and SAICAR, can act as signaling molecules. I extensively illustrate the nature of a metabolic signaling hub using the specific example of the Target of Rapamycin Complex 1 (TORC1), and amino acid sensing. A highlight is the emergence of the lysosome/vacuole as a metabolic and signaling hub. Finally, the need to expand our understanding of the intracellular dynamics (in concentration and localization) of several metabolites, and their signaling hubs is emphasized.

Keywords: *Metabolic signaling, Acetyl-CoA, S-adenosyl methionine, TORC1, mTORC1, Amino acids, Lysosome, Vacuole*

1 Introduction

The need to understand the concept and process of cell growth and proliferation piqued the interest of biologists like Rudolf Virchow over a century ago, and continues driving the curiosity of biologists today.[1–3] In simple, unicellular model systems, an emphasis has been to understand growth as a program in response to nutrient availability.[4–6] In mammals, the drive to understand growth has come largely from our desire to understand cancer.[2] As our understanding of growth processes expanded, an interest in the various signaling systems controlling growth dramatically expanded.[2]

At the heart of the process of cell growth are the metabolic processes in a cell that enable growth, ranging from making energy in the form of ATP, to providing lipids, amino acids and nucleotides along with various co-factors.[7] Metabolism has been studied extensively for decades. However, a recent surge in interest in metabolism has come with the realization that metabolic transformations are a key hallmark of cancer.[2, 3, 8] With that understanding, an old phenomenon called the "Warburg effect" has come back into prominence. The Warburg effect, described in the 1920s by Otto Warburg, came from the observation that cancer cells took up enormous amounts of glucose, and instead of efficiently metabolizing glucose through the TCA cycle, fermented glucose to produce lactate.[9, 10]

TCA cycle: The TCA cycle (tricarboxylic cycle), also known as the citric acid cycle, or Krebs cycle, is a major biochemical pathway used by all aerobic organisms to generate energy by oxidizing acetyl-CoA (derived from various sources) into carbon dioxide and ATP, which is the biological form of energy.

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Springer IISc Press

With the now resurging interest in metabolism driving cellular transformation, there are constant efforts to explain how the Warburg effect is achieved, and what it enables cells to accomplish.[3, 11] And it is perhaps due to this resurgence in metabolism that the intimate interplay and cross-regulation between metabolism and signaling are emerging.[12] While both are critical to the process of regulating cell growth, metabolism and signaling had somehow diverged to become important but separate.[12, 13] This is despite the now vast understanding of how signaling regulates metabolic homeostasis. It is increasingly clear that while signaling directly regulates metabolism, metabolism can also directly regulate signaling, in an effort by cells to accomplish regulated growth. This review will attempt to explain concepts in metabolic signaling, emphasizing both aspects from metabolism and signaling in this process.

Allostery: or allosteric regulation is the regulation of the activity of a protein by any effector molecule, by binding the protein at a site different from the protein's active site.

An important area in the field of "metabolic signaling" will not be explored in this review; that of systemic control of signaling through growth factors, tyrosine phosphatases, and nuclear hormone receptors. All of these are exceedingly important to metazoan function, and their growth and development in response to overall nutrient status. The general principles by which they operate have been extensively reviewed (for example in [14–17]). In contrast, the purpose of this review is to illustrate core principles of how intracellular metabolic states are directly sensed, and how these regulate outputs related to *that specific* metabolic cue. Such principles and processes are typically conserved from simple, unicellular eukaryotes like yeast through metazoans.

While studying metabolic signaling, it is important to note that the correlation between gene expression and metabolism is relatively poor in eukaryotes. Even in organisms like yeast, where general correlations between transcription and translation are reasonable,[18] metabolite levels and the expression levels of genes encoding enzymes that synthesize or degrade these metabolites are not well correlated,[19–24] and better correlations have not yet been observed in mammals or other metazoans. Therefore, some traditionally used concepts in signaling (emerging from understanding gene regulation) are perhaps a little less useful in understanding metabolic signaling. In many ways, to understand metabolic signaling, it requires going back to the decades-old, but now under-appreciated concept that metabolic flux is largely regulated by a combination of mass action reactions, and allosteric regulation achieved by small molecules (metabolites), and regulatory, post-translational modifications.[7]

Transcription: Initial step in gene expression, where DNA is copied onto messenger RNA (mRNA), by an enzyme, RNA polymerase.

Translation: The process by which mRNA is decoded to produce polypeptides (proteins), by a large molecular machine (containing proteins and RNAs), the ribosome.

2 Allosteric Regulation at the Core of Both Metabolic Regulation and Signaling

Regulation of cellular function can be achieved by multiple mechanisms, the most extensive of which involve remodeling global outputs by transcriptional and translational regulation. However, as early studies of what later became signaling reveal, one of the most effective ways to achieve regulation is by allosteric modulation by small molecules.[25, 26] While the concept of allostery is simple, it can explain how a molecule can inhibit or activate a protein without binding to an active site.[25] Indeed, this is how general regulation is achieved in most metabolic pathways, [7] and a majority of metabolic outputs can be explained by a combination of allosteric regulation and mass action.[7, 24] Interestingly, the principles of allosteric regulation, discovered and defined using enzymatic systems that form the core of metabolism, are what were originally used to understand and build the principles of signal transduction.[27, 28] Allosteric regulation provides multiple advantages to enable regulation. It is a mechanism to directly sense amounts of a small molecule, it can be effective rapidly or slowly (depending upon the binding affinity of the molecule with its receptor), and it can be easily reversed.[25, 26] These are often the key requirements for signaling systems, particularly those responding to the availability of nutrients (or cues derived from nutrients).

Three key discoveries that led to the core concepts of signaling came from studies of metabolism and allosteric regulation; these were the discovery of reversible protein phosphorylation as a means to regulate enzyme activity, protein kinases and protein phosphatases.[29–32] All of these led to the eventual framework of a "writer–reader–eraser" module that defines cell signaling.[33] Remarkably, all of these discoveries came from studying the activation of glycogen phosphorylase (a key enzyme in glycogenolysis), when tissues were stimulated by hormones like epinephrine.[32] The other concept that emerged from studies of metabolism, and became mainstream in signaling is that of a negative feedback loop. End product "feedback inhibition" occurs when an accumulated metabolic product inhibits the

enzyme making this molecule, and this is analogous to the inhibition of a signaling system by a final effector in the pathway. Viewing signaling historically, when the building blocks of what became signaling were still being discovered, it was clear that signaling and metabolism are intimately connected.[29–32, 34, 35] Yet over time this connection was lost, with signaling and metabolism becoming important but separate entities, and this connection is only now being re-appreciated.[12, 13] It is also now clear that this is not a one-way road, but that just like signaling regulates metabolism, metabolism reciprocally regulates signaling, and these can be both immediate as well as long-term homeostatic effects.

To understand broader principles in metabolic signaling, there are two concepts that are important. The first is in understanding what a "metabolic sensor" might be, and how to conceptualize a metabolic sensor. The second is expanding the role of what are typically viewed as central metabolites, to signaling molecules or systems. In this review we explore both aspects, and delve deeper into amino acid sensing and signaling, where much has been established, but many important questions remain unanswered.

3 Building a Metabolic Sensor-AMPK and "Energy Sensing"?

The term "metabolic sensor" is now an abused term in the scientific literature, to the extent that any protein that binds a central metabolite can be dubbed a metabolic sensor. By that definition, a plurality of the proteome, which includes large numbers of proteins that have metabolites like NAD+, thiamine, cobalamine, NADPH, or sugars as cofactors, would all be metabolic sensors, and this premise is obviously absurd. It is, therefore, far more useful to conceptualize metabolic sensors from core principles of metabolic regulation. If a cell has a specific metabolic state which is determined by the accumulation of a key metabolite, a bona fide metabolic sensor would directly bind to this metabolite, be activated (or inhibited) by it, and direct a response (typically through a signaling cascade) resulting in the eventual utilization of that metabolite, a corresponding transformation of the cell, and the restoration of metabolic homeostasis. While this is not an exclusive definition of a metabolic sensor, it is a particularly useful one to illustrate key aspects of metabolic sensing. An exemplary example of such a metabolic sensor is the adenosine monophosphate activated protein kinase, AMPK.[36–38]

The ancestral role of AMPK is undoubtedly carbon or energy sensing, [36, 39] as seen first in pioneering studies from *Saccharomyces cerevisiae*, where the AMPK ortholog (Snf1) regulates responses to glucose starvation, and controls the expression of enzymes that enable yeast to switch from glucose fermentation to oxidative phosphorylation.[5, 40] But its role as a metabolic sensor was revealed only when its biochemical mechanism of action was elucidated. The AMPK protein is a heterotrimer, comprised of three different, evolutionarily well-conserved proteins. At the core is a catalytic kinase subunit, while the other two, the β and γ subunits, are allosterically regulated subunits. The γ subunit contains multiple Bateman domains,[41] forming four cavities capable of binding ligands containing adenosine moieties. Interestingly, these domains are capable of binding ATP, ADP and AMP.[42] When cells are "low energy", there is a decrease in the total ATP concentrations, with an increase in ADP or AMP concentrations. Therefore, AMPK activation involves allosteric activation of AMPK by ADP or AMP, depending upon the severity of energy stress. If, instead of ATP, the third cavity is bound by ADP/AMP, a specific threonine residue on AMPK is phosphorylated, resulting in an eventual 100-fold activation of the protein. AMPK can be further activated another tenfold allosterically during very severe ATP depletion, when another AMP replaces an ATP that was formerly bound. This can be easily reversed when ATP levels are restored, again by allosteric regulation, when ATP replaces ADP or AMP. These mechanisms of AMPK activation are extensively reviewed in.[42]

AMPK regulates metabolism, mitochondrial biogenesis and lipid metabolism through a number of direct and indirect mechanisms. In mammals, upon activation AMPK phosphorylates substrates that eventually result in an increase in glucose transporters and glucose uptake, glucose oxidation, and fatty acid oxidation.[38, 39] In yeast, similar processes including the activation of mitochondrial biogenesis and function are observed.[5] However, it is unclear if the ATP/(AMP or ADP) ratio directly activates AMPK (Snf1) in yeast, or if it is somehow activated directly by low concentrations of glucose, [5] although the metabolic outputs of Snf1 closely resemble those of mammalian AMPK. The diverse mechanisms by which AMPK regulates metabolic outputs to restore ATP, as well as the nature of AMPK substrates are all subjects of numerous excellent reviews.[5, 36–39, 42]

Finally, illustrating how closely "energy sensing" by AMPK is connected to growth outputs and other metabolic sensing, namely amino acid sensing, recent studies illustrate how AMPK and the amino acid sensing Target of Rapamycin Complex 1 (TORC1) are reciprocally regulated. When carbon sources are limited, elevated AMP activates the AMPK, and also recruits it to the amino acid sensing complex that activates TORC1. Here AMPK phosphorylates two important regulators of the TORC1, Raptor and the TSC1:TSC2 complex (discussed later), and results in an inactivation of TORC1.[43, 44] The sensing of amino acids through the TORC1 is described extensively in a subsequent section of this review.

4 Metabolitesas Signaling Molecules

As the example of AMPK shows, metabolic sensors can typically sense key metabolites and metabolic states, and regulate multiple outcomes. However, it is also possible to conceive of certain metabolites themselves as signaling molecules. These may even be possible in metazoans, where otherwise growth factor signaling plays such a prominent role in maintaining metabolic homeostasis. So how might we be able to think of metabolites as signaling molecules, and what kinds of evidence are emerging for this?

A central metabolite rapidly emerging as a critical signaling molecule is acetyl-CoA. This is normally thought of as a molecule at the heart of energy metabolism, feeding into the energy producing oxidative-phosphorylation cycle, or entering the fatty acid biosynthesis pathway, and also serves as an allosteric regulator of a number of enzymes.[7] It is now apparent that acetyl-CoA has major signaling roles in eukaryotic cells, through the process of protein acetylation.

Acetyl-CoA: A central metabolite critical for carbohydrate, lipid and protein metabolism. Acetyl-CoA is oxidized in the TCA cycle, eventually to carbon dioxide and water, with the formation of 11 molecules of ATP, and one molecule of GTP per acetyl group.

4.1 Acetyl-CoA and Lysine Acetylation Regulating Signaling and Gene Expression

The process of protein acetylation involves the transfer of an acetyl group derived directly from acetyl-CoA, to lysine residues (at the N^e amine) on proteins, by acetyl transferase enzymes. This modification is entirely reversible, and deacetylation is carried out by deacetylases. This is quite analogous to classic signaling post-translational modifications, such as reversible phosphorylation.[29] Such a modification can alter the activity or stability of a protein, or its localization, or association with other proteins, just as any other signaling modification could. When thinking about phosphorylation, one is not very concerned about the availability of the phosphate donor, ATP. In stark contrast, with acetylation, it is increasingly evident that protein acetylation, and the nature of proteins being acetylated, is very closely tied to acetyl-CoA concentrations and cellular metabolic states.[45–51]

The most direct lines of evidence illustrating how acetyl-CoA levels regulate cellular outcomes by altering acetylation processes comes from studies in yeast.[52, 53] Just as yeast enters into a growth phase, acetyl-CoA levels rise, fuelled by metabolic processes that allow acetyl-CoA formation. Concurrent with that, there is a dramatic increase in the acetylation of histones positioned specifically at the promoters of "growth-specific" genes, and this histone acetylation activates those genes.[53] A specific acetyltransferase, Gcn5, which is a part of the SAGA complex, mediates this growth-specific acetylation.[53] Therefore, the availability of the metabolite, acetyl-CoA, regulates growth. Furthermore, the availability of acetyl-CoA appears to directly drive entry into the cell division cycle by activating the transcription of the central G1 cyclin, CLN3, by acetylating histones at the CLN3 promoter.[52] The scope of these studies from yeast have now been dramatically expanded, with recent studies showing how pools of acetyl-CoA, derived from acetate (and not glucose) can drive cell proliferation in a variety of carcinomas, from hepatocarcinomas to glioblastomas.[54, 55]

Mitochondria: A major, membrane bound organelle present in eukaryotic cells, which plays a central role in metabolic regulation, generates ATP, and precursors or intermediates for amino acids.

4.2 Pools of Acetyl-CoA

It is important to note that acetyl-CoA is present in multiple pools within cells (Fig. 1). This molecule is ideal to exist as pools, because it is membrane impermeable. The molecule comprises of an acetyl group covalently linked to coenzyme A, made from vitamin B5.[7, 45, 50] This means that it is likely to either remain where it is made, or needs to be actively transported to other locations. Thus, a major pool of acetyl-CoA is found in the mitochondria, where it is made as a first step in the TCA cycle.[7, 12, 50] However, this pool of acetyl-CoA is distinct from the cytosolic or nuclear pools of acetyl-CoA,[12, 50] although acetyl-CoA can be converted to citrate, enter the cytoplasm and be reconverted to acetyl-CoA (Fig. 1). Correspondingly, a variety of different enzymes enable the formation of acetyl-CoA, from different sources (Fig. 1). These include ATP-citrate lyase, or acetyl-CoA synthetase, or the pyruvate dehydrogenase complex in the mitochondria. Interestingly, the

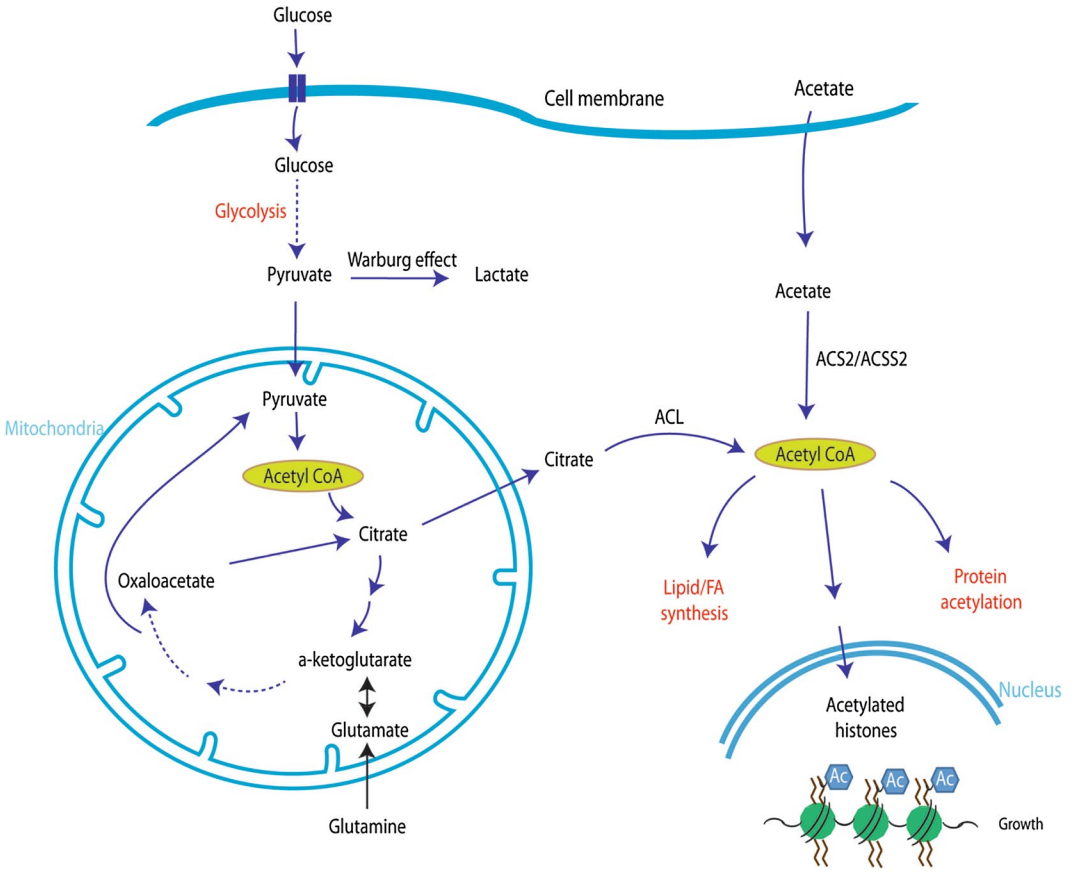


Figure 1: Pools of acetyl-CoA, and acetyl-CoA in gene regulation. Acetyl-CoA is typically made from glucose, but can also be made from other carbon sources such as acetate. Acetyl-CoA is restricted to multiple pools within the cell, particularly mitochondrial pools and nuclear-cytosolic pools, due to its membrane impermeability. Nuclear pools of acetyl-CoA are used to acetylate histones and can lead to substantial changes in gene expression, controlling growth. Cytosolic pools of acetyl-CoA are used towards fatty acid synthesis, or to acetylate a wide range of proteins and thereby regulating their activity and function

cytosolic/nuclear pools in particular appear to change quite dramatically, depending upon nutrient availability and fed/fasted states of cells.[49, 50] In well-fed conditions, excess acetyl-CoA in the mitochondria is shunted out to the cytoplasm, and diverted to fat synthesis and storage. Thus, the cytosolic and nuclear pools can be utilized for the variety of acetylation reactions with their consequences, as we have just discussed. This rate limiting nature of pools of acetyl-CoA for protein acetylation, making the signaling aspect of acetyl-CoA unique and directly linked to metabolic states, is a rapidly emerging area of current research.[12, 45, 46, 50, 56]

Protein acetylation in regulation In this context, advances in analytical approaches, particularly mass spectrometric methods as well as the development of acetyl-lysine specific antibodies, have advanced the study of protein acetylation as a major, regulatory post-translational modification.[48, 57, 58] These studies have revealed that lysine acetylation is not limited to histones, but to a range of cytosolic and mitochondrial proteins as well, [48] consistent with our knowledge of acetyl-CoA pools. Like other post-translational modifications, acetylation also controls gene expression (directly, through histone acetylation), protein–protein interactions, or even the direct regulation of metabolism through the acetylation of several metabolic enzymes.[59–62] Intriguingly, these studies also revealed that a majority of the enzymes involved in intermediate metabolism, particularly glycolysis, appear to be acetylated, and that the activity of these enzymes are altered by acetylation. Also intriguingly, while modifications like phosphorylation occur typically in unstructured regions of proteins, acetylation appeared to occur in highly structured regions in proteins.[48, 58] These are still early days in understanding how the process of protein acetylation acts as a signaling

modification, and much needs to be discovered. We can already expect that direct parallels with phosphorylation may not translate to acetylation, especially given the very large numbers of kinases present in all eukaryotes, compared to a much smaller set of acetyl transferases. Also of note, understanding the regulation of acetylation by deacetylases, both histone deacetylases as well as the sirtuin class of deacetylases, are areas of increasing interest.[12, 48, 63]

Another post-translational modification dependent on a central metabolite, that is emerging as a regulator of multiple signaling pathways is protein methylation.[51] Methylation of lysine or arginine residues requires *S*-adenosyl methionine (SAM) as a substrate, and is carried out by a range of protein methyl transferases.[64] These modifications are also reversed by demethylases, and the system functions similar to the widely prevalent kinase–phosphatase networks, and it is now clear that methylation is a major signaling regulator.[33] To fully establish that SAM itself is a "signaling" metabolite, and not just a central metabolite, it would be ideal to observe different protein methylation outputs dependent on SAM availability at local pools. However, while the similarity to acetylation and its dependence upon amounts of a central metabolite (acetyl-CoA) are immediately apparent,[51] the relationship of methylation to actual pools of *S*-adenosyl methionine, and varying concentrations of SAM have still not been carefully explored. This is in part because of challenges in measuring pools of SAM within the cell, although decades-old studies from yeast suggest SAM to be sequestered or stored within organelles like the vacuole.[65, 66] However, recent reports in yeast suggest the example of the methylation status of protein phosphatase PP2A, which is regulated by a specific methyltransferase, [67–69], to be highly dynamic, and dependent upon SAM availability.[70, 71] These studies also hint at differential substrate specificities of PP2A controlled by SAM availability.[70, 71] Given the importance of PP2A as a major signaling regulator, [72] this can have dramatic consequences on modulating signaling outputs. Therefore, as a candidate for a bona fide metabolic signaling molecule, SAM appears to be prime.

Several other examples of metabolites as signaling molecules are now emerging, following the themes we have discussed, [73] and their roles as signaling molecules are areas of active interest. There is a curious case where a well-known metabolic enzyme appears to also be moonlighting as a protein modifying/signaling enzyme. The "Warburg effect", where cells have elevated rates of glucose uptake and glycolysis, is a classic hallmark of many cancers.[3] Some years ago, it was observed that a well-known glycolytic enzyme, pyruvate kinase (PKM), was sometimes expressed in tumor cells as splice-variant called PKM2, and this protein was responsible for the transformation into a highly glycolytic state.[74] Since then there has been an explosion of studies attempting to understand how PKM2 is regulated, by glycolytic metabolites, [74, 75] protein modifications, [75, 76] or intermediates in nucleotide metabolism.[77, 78] While it is very unclear if PKM2 truly activates tumor formation, [75, 79] interestingly, it appears that whatever property PKM2 has to regulate growth comes from a possible, unusual role as a protein kinase [80, 81] and not its primary, well studied role in metabolism converting phosphoenol pyruvate to pyruvate. This atypical activity appears to allow PKM2 to localize in the nucleus and regulate classic "growth signaling" pathways, including the activation of the myc transcription factor, explaining how it might function to regulate tumor formation. Also interestingly, this role of PKM2 to function as a protein kinase appears to be allosterically regulated, particularly by SAICAR (an intermediate in nucleotide metabolism).[77] The allosteric activation of PKM2 by SAICAR appears to induce the protein kinase activity of PKM2, leading to it phosphorylating many protein kinases, including Erk1/2, which in turn phosphorylate PKM2 and further activate its protein kinase activity in a classic feed-forward loop, potentially driving proliferation.[77] While the in vivo importance of this role of PKM2 is yet to be elucidated, this discovery, requiring the use of classic methods in biochemistry and metabolism, highlights conceptual possibilities with metabolic signaling, which are still barely explored.

5 Amino Acid Sensing, TORC1, and the Lysosome/Vacuole as a Metabolic Signaling Hub

Conventionally, amino acids are thought of in the context of protein synthesis, and cell growth. However, amino acids, like Acetyl-CoA and SAM, are not only central metabolites critical to the process of anabolism and growth, but also fit many criteria of conventional signaling molecules. Indeed, we now know that amino acids are not uniformly present in cells, but are present in pools,[65, 82] and that these pools are dynamic and tightly regulated.[66, 82, 83] Amino acids also serve as precursors to carbon metabolism, and nucleotide biosynthesis.[83] Thus, amino acids are not only central to cell growth, but also are distinct in their

S-adenosyl methionine: or SAM is an important metabolite, which serves as the primary methyl group donor in almost all methylation reactions in cells. SAM is made from the amino acid methionine, and ATP.

Kinase and phosphatase: These are enzymes which can covalently add (kinase) or remove (phosphatase) a phosphate group onto serine, threonine or tyrosine residues present on proteins.

Amino acids: Important metabolites that contain an amine and a carboxylic acid functional group, along with side chains specific to each amino acid. Amino acids form poly-peptide chains to become proteins, which perform most functions within cells.

versatility and multitudes of outcomes. It, therefore, seems reasonable to expect that cells will have sophisticated ways to sense amino acids, and signal responses to amino acid availability.

In order to understand amino acid sensing and signaling, we need to understand an underappreciated organelle, the lysosome (or the vacuole in plants and fungi). The lysosome was first observed by Christian de Duve in 1955 as a membrane bound organelle, filled with hydrolases and other enzymes that could break down proteins, carbohydrates and lipids.[84, 85] Because of these obvious enzymatic activities, the lysosome was declared the "garbage bin" of the cell. It was only with the discovery of autophagy in yeast by Ohsumi that it rapidly began to emerge that the lysosome had sophisticated roles in maintaining metabolic homeostasis in cells.[65, 66, 82] After it was established that the vacuole functioned to compartmentalize important metabolites, it became clear that this role was active and regulated, and not passive.[65, 66, 82, 86] What surprisingly emerged was that amino acids were not metabolites that freely floated within the cytoplasm, but that many amino acids existed in pools within cells. The lysosome/vacuole was central to maintaining these distinct pools of amino acids, serving as a storehouse for them, and making them available in the cytosol as needed by the cell. Many early studies, especially in yeast, showed that several amino acids including arginine, histidine, and lysine accumulated to extremely high concentrations in vacuoles, while others like aspartate, leucine, isoleucine and glutamine were retained primarily in the cytoplasm, with glutamine also translocating to the mitochondria to fuel the TCA cycle.[66, 87–90] Additionally, decades-old studies showed that critical metabolites derived from methionine metabolism, including *S*-adenosyl methionine, are actively transported into the vacuole and stored.[91–93] Surprisingly, we know very little about the regulation of these dynamic pools of amino acids. This area of research has remained largely ignored for decades, but with our constantly improving understanding of how amino acids are sensed in cells (explained subsequently), there is an emerging interest in understanding the regulation of amino acid pools. This will be central to our eventual understanding of amino acid homeostasis, and the translation and metabolic outputs of amino acids.

Collectively, the lysosome/vacuole appears to not only alter the rates of metabolic processes occurring elsewhere in the cell, but also acts to communicate this metabolic information to metabolic sensing hubs, which we will explore shortly. It is, therefore, now obvious that we need to obtain a better understanding of the functional organization of the lysosome, as well as acquire at least a basic idea of its structural and functional components. We also have a minimal knowledge of how the composition and function of the lysosome changes in cells under different metabolic states, but understanding this becomes critical for our understanding of metabolic homeostasis. Particularly, understanding lysosome organization and function is central to our understanding of how amino acids are sensed, and signal.

Traditionally, our understanding of how cells perceive changing nutrient status, and integrating this with metabolic outputs come from a conventional view of signaling, where a ligand, such as a growth factor, binds an extracellular receptor, and activates a signal transduction cascade. A typical example would be the insulin/insulin receptor-mediated growth factor signaling.[15, 17] Contrastingly, we understand less about how intracellular metabolic signals are perceived, and how they signal within the cell. Given that most amino acids are regulated tightly *inside* the cell, and the lysosome appears to be central to this process, it is this internal signaling that becomes relevant to amino acid signaling. And the key player in this process is a signaling hub and metabolic master regulator called the Target of Rapamycin Complex 1 (TORC1) in all eukaryotes, or mTORC1 in mammals.[94–96]

Autophagy: is an orderly process within cells resulting in the systematic degradation and recycling of cellular components, typically during starvation.

Lysosome/vacuole: A major, membrane bound organelle present in eukaryotic cells, called the vacuole in plants and fungi, and lysosome in other eukaryotic cells. This serves as a storehouse for nutrients, and a hub for protein turnover and signaling.

5.1 TOR and its Mode of Action

Today, it is widely appreciated that the TORC1 is central to a cell's ability to adapt and respond to multiple environmental stimuli. The TORC1 is conserved across eukaryotes from yeasts to mammals, and controls growth and metabolism.[5, 94–96] TORC1 is itself directly controlled by growth factor signaling, carbon sources and "energy levels" by the AMPK, oxygen availability, and most strikingly, by amino acids.[5, 95, 97] Yet the discovery of such a major regulator of cell function was largely serendipitous.

25 years ago, Joseph Heitman and Michael Hall decided to use budding yeast to determine the target of the immunosuppressant cyclosporin, along with trying to identify the target of a new immunosuppressant called rapamycin, both of which were originally isolated from soil dwelling bacteria. Their idea was that the original purpose of these molecules, produced by microbes, could not be immune suppression or aiding organ transplants, but would be to inhibit the growth of other, competing microbes. This

idea, and approach to use *S. cerevisiae* turned out to be wildly successful, and a tour de force of using genetic approaches to identify the target of a drug. Heitman and Hall isolated *S. cerevisiae* strains that developed resistance to rapamycin, and identified the genes responsible for conferring resistance.[98] One gene was a protein called FKBP12, and the others were two genes, which they called *Tor1* and *Tor2*, or Target of Rapamycin 1 and 2.[98] A few years later, David Sabatini and Solomon Snyder, as well as Stewart Schreiber's group, both independently identified the target of rapamycin in mammalian cells.[99, 100] While many names were in use for this protein initially, the final consensus settled on mTOR, or mammalian target of rapamycin. It became immediately apparent that the mammalian gene was the ortholog of the yeast genes originally discovered, and that TOR was a protein kinase, although related to the PI3 lipid kinase family.

But the key was identifying what TOR did. Elegant studies again in yeast showed that TOR was a central controller of cell growth, with cells arresting in G0 when grown on poor nitrogen sources.[101–103] Subsequent studies show that TOR regulates growth primarily by controlling protein translation directly, through the activation of the ribosomal S6-kinase (S6K) by phosphorylation, [95, 102, 104] as well as by suppressing the eukaryotic translation initiation inhibitor, 4E-BP, by phosphorylation.[105] The connection to amino acids were first hinted by the yeast studies, connecting nitrogen availability to TOR, and subsequently genetic studies in *Drosophila* showed that TOR loss of function closely mimicked amino acid starvation in flies.[106, 107] Finally, Hara et al.[108] showed in mammalian cells that amino acid starvation dramatically reduced S6K and 4E-BP phosphorylation, directly connecting TOR function to amino acid availability. It is now apparent that amino acids are the most critical signals to activate TOR, and that none of the other activators of TOR activity can fully activate TOR in the absence of amino acids.[97] Furthermore, in several simple systems such as budding yeasts, where growth factor based regulation is absent, full TOR activity can be seen in the presence of abundant amino acids.[5] All of these data suggest that the primordial role for TOR is amino acid sensing, and coupling amino acid availability to growth outputs.

We now have a reasonable understanding of the composition of the core TOR complex. In yeast and mammals, the TORC1 includes not just the TOR kinase, but also Kog1 (in yeast), or its homolog RAPTOR (mammals), and Lst8.[5, 95, 102, 109] In response to amino acids, we also now have an increasingly sophisticated understanding of what TOR does.[5, 95, 97, 110, 111] In *S. cerevisiae*, the primary downstream responses are regulated by a protein kinase Sch9, which also acts as a ribosomal S6K, [104] as well as the protein phosphatase PP2A.[5] TOR directly phosphorylates and activates Sch9, which has a number of downstream effects leading to increased ribosome biogenesis, translation activation, increased anabolism and ATP and nucleotide synthesis to fuel growth.[5, 102] The exact mechanisms by which TOR regulates PP2A are still unclear, but appear to function largely through the phosphorylation of Tap42, which associates with PP2A proteins and directs or alters phosphatase substrate specificity.[5] The two best-characterized substrates of mammalian TORC1 are the ribosomal S6K and 4E-BP1, which mediate its role in translation activation. Again, the distal readouts of TOR activation are increased protein synthesis, ribosome biogenesis, and the regulation of cell size. TOR also activates multiple transcriptional outputs, which also enable growth related processes. Importantly, TOR regulates multiple metabolic outputs including amino acid biosynthesis and glucose homeostasis.[5, 95, 97, 110, 111] Additionally, in metazoans, TOR also regulates adipogenesis, fat metabolism and obesity, and this is an area of intense interest.[112]

We are still left with many questions asking how TORC1 senses amino acids and is activated by them. The TOR kinase itself does not bind to amino acids, nor do the core components of TORC1. However, our understanding of how cells sense amino acids through TOR is systematically expanding, and several themes are emerging in this context. First, the localization of TORC1 has been the key to understand amino acid sensing. Second, as multiple players upstream of TORC1 that regulate amino acid sensing have been identified, it appears that there may not be a single "amino acid sensor" in eukaryotic cells, but multiple components which allow specific and distinct responses to different amino acid groups.

5.2 TORC1 Localization and Amino Acid Sensing at the Lysosome

The localization of TORC1 has been instrumental in helping build our understanding of amino acid sensing. Early studies in yeast showed that a large fraction of the cellular pool of the TORC1, particularly Kog1 and the Tor kinases, were localized on the vacuolar membrane.[102, 109, 113, 114] These studies hinted that vacuolar localization of the TORC1 might somehow be important for amino

acid sensing. Subsequently, several landmark studies in mammalian cell lines, using conditions of amino acid withdrawal, or addition, showed a very dynamic, amino acid dependent redistribution of the TORC1 in cells.[115, 116] Initial studies only showed that upon amino acid stimulation, TORC1 redistributed into vesicular puncta in cells, but it soon became clear that these puncta were the surface of lysosomes.[115, 116] In particular, it was striking that this localization of TORC1 on lysosomes required amino acid stimulation, but not other stimuli that activated TORC1 such as growth factors.[115] Importantly, it has also become clear that this recruitment of TORC1 to the lysosome is critical for the amino acid dependent outputs of TORC1.[115–117]

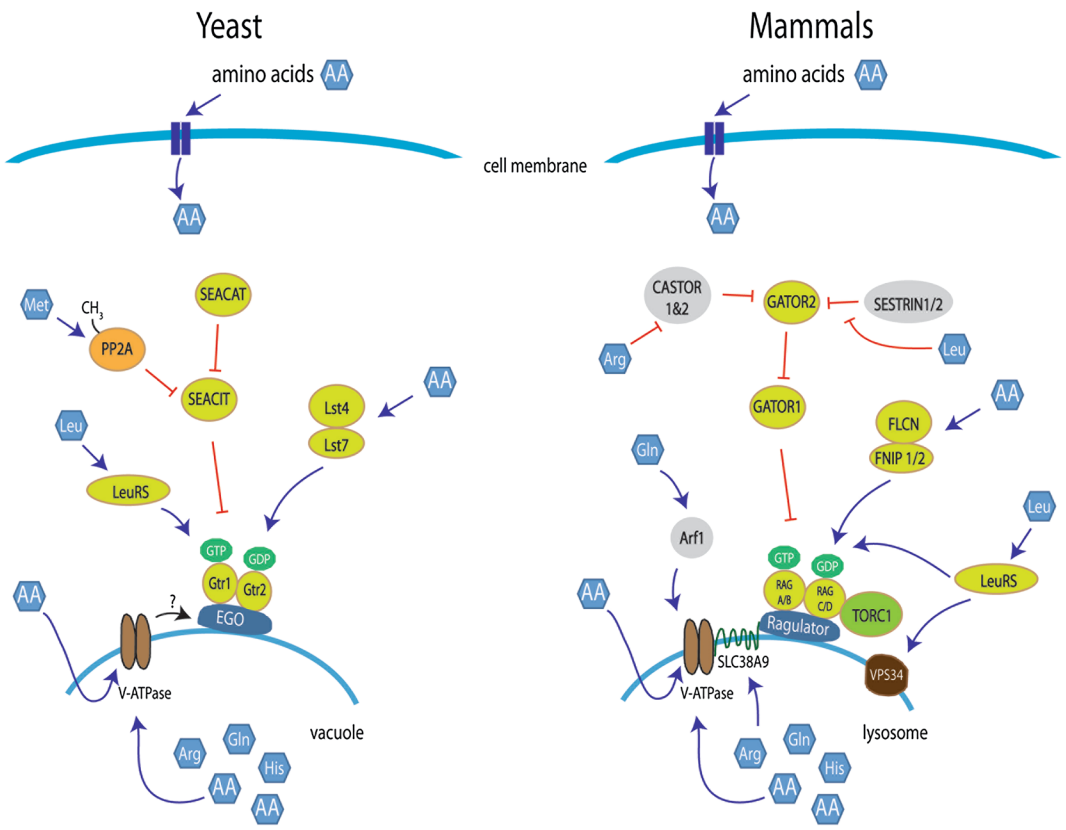
Even as this close connection of TORC1 and amino acid sensing to the lysosomal surface has developed, in parallel several missing pieces of components that enable TORC1 activation by amino acids are emerging. It was apparent early on that Kog1/RAPTOR is essential for all amino acid responses of TORC1.[103, 108, 115, 117] RAPTOR is a very large protein with no catalytic activity itself, but can act as a very effective scaffold to bring together multiple proteins. The emerging model is that RAPTOR can dynamically hold together multiple proteins to potentially sense amino acids and transmit this information to effectively activate the TORC1, [103, 108, 115, 117] and that this involves several players on the vacuole/lysosome surface. How this happens, and specifically for single or multiple amino acids, has become an important question to answer. For the emerging discoveries in this area, it has been useful to think of the types of amino acids TORC1 respond to, and the identity of the proteins that enable amino acid sensing.

So what amino acids does TORC1 respond to? Early work in mammalian cell lines, xenopus oocytes and other models suggested that TORC1 activation was amino acid dependent but particularly sensitive to branched chain amino acids, especially leucine.[108, 118–120] This drove much of the emphasis towards finding a "leucine sensor". However, multiple recent studies show that TORC1 responds very effectively to other amino acids, including glutamine, [121–124] arginine, [123–126] serine, [123, 124] and methionine.[70, 124, 127] A recent reconstitution of TOR activity in vitro clearly shows a remarkably graded response of TOR activity towards different amino acids, with priming of the TORC1 achieved by asparagine, glutamine, threonine, arginine, glycine, proline, serine, alanine and glutamic acid, and activation potently achieved by not just leucine, but also methionine, isoleucine and valine.[124] All these data suggest the possibility of multiple "amino acid sensors" upstream of the TORC1. Independently, the identification of key upstream components responsible for TORC1 activation also suggests the same conclusion. Figure 2 illustrates the various ways by which TORC1 can be activated or controlled by amino acids.

The discovery of a family of small GTPases, which help bring together lysosomal/vacuolar localization, amino acid sensing and TORC1 activity, has been central to placing together a mechanism of TORC1 activation by amino acids. These GTPases, which called the RagA/B and RagC/D in mammals [128] and Gtr1/Gtr2 in yeast, [117] were discovered independently and simultaneously in these organisms.[117, 128] The RAG or Gtr proteins function as heterodimers, and for full amino acid activation, cells require RagA/B or Gtr1 to be loaded with GTP, while RagC/D or Gtr2 is loaded with GDP.[117, 128] This active form of the heterodimer assembles upon stimulation with amino acids, directly binding TORC1 through Kog1/RAPTOR.[109, 117, 128, 129] Thus, this entire complex and the process of assembly and activation of TORC1 at the lysosome upon amino acid stimulation is highly dynamic. This dynamic activation of the Gtr1/2 or RagA/B and RagC/D proteins depends on functionally analogous complexes on the lysosome/vacuole surface in yeast and mammals, called the EGO complex or RAGULATOR, respectively.[109, 117, 128, 129] These complexes contain other proteins (in addition to Gtr1/2 or RagA/B and RagC/D), which not just help assemble these GTPases, but also appear to have guanine nucleotide exchange (GEF) activity, [130] and enable an interaction with a key component for amino acid sensing, the vacuolar ATPase (Fig. 2),[116, 131, 132] all of which are discussed later.

5.3 Regulating the Rag GTPases

Studies with the RAG/GTR GTPases immediately revealed that the nucleotide binding state of these GTPases is critical to assemble their heterodimers, and mediate full activation of TORC1 upon amino acid stimulation, [109, 117, 128] and the Rag-RAPTOR interaction is critical for activation.[111] Thus understanding how the nucleotide binding states of the RAG GTPases are regulated becomes critical to understanding amino acid dependent activation of TORC1. Multiple independent studies, from mammalian cells and flies, identified the Folliculin (FCN) tumor suppressor, with its binding partner FNIP1 or 2, as positive regulators of RAG function.[111, 133] This complex



Orthologs (different nomenclature)

Yeast	Mammals
SEACIT (Npr2, Npr3, Iml1)	GATOR1 (Nprl1, Nprl2, DEPDC5)
Gtr1, Gtr2	RAG A/B, RAG C/D

Functional equivalents

Yeast	Mammals
EGO	RAGULATOR
SEACAT	GATOR2
Lst4/ Lst7	FLCN/ FNIP 1/2

Figure 2: Amino acid sensing and signaling by TORC1. In all eukaryotes, amino acid sensing is carried out by several proteins which all regulate the activity of the TORC1. The figure illustrates largely conserved modes of the amino acid sensing and signaling by the TORC1 in yeasts (***S. cerevisiae***) and mammals. Note that the vacuole or lysosome membrane is key to proper amino acid sensing, by holding together the TORC1. Also, the core amino acid responding complex, the TORC1, is well conserved between these organisms, however some components are not. There do typically exist functional equivalents across species, which function in similar ways. The TORC1 responds to a variety of amino acids, and it is still not clear how important the v-ATPase is for all amino acid responses, although it is critical for many

appears to stimulate the GTPase activity of RagC/D. Analogously, in yeast the Lst4–Lst7 proteins plays similar roles, shuttling to the vacuole during amino acid availability, and stimulating Gtr2 GTP hydrolytic activity and thereby activating TORC1.[109, 127] While all of these systems to activate the RAG GTPases are very sensitive to leucine, they do not seem to be uniquely activated by a single amino acid, but instead are important for all amino acid sensing through TORC1.

However, an unexpected regulator of the RAG GTPases appears to be the leucine tRNA synthetase LeuRS. Two groups independently identified this role for the LeuRS, in yeast and mammals. In yeast, the LeuRS was found to bind Gtr1, and that mis-charged LeuRS (which is present during leucine starvation) had reduced interaction with Gtr1, resulting in lower GTP loading of Gtr1 and, therefore, reduced TORC1 activity.[134] In contrast, in mammalian cells,

LeuRS was recruited to the lysosomal membrane during lysine stimulation, and enhanced GTP hydrolysis of the RagC/D (the orthologs of Gtr2), thereby activating the Rag heterodimer and, therefore, TORC1 activity.[135] While both studies convincingly show that mischarged LeuRS is an important regulator of TORC1 function through the Rag/Gtr proteins, it seems perplexing that a system superbly conserved across evolution appears to have very distinct modes of regulation to achieve the same outcome. Only a more systematic biochemical approach leading to mechanism can explain how this is possible.

The other side to the regulation of Rag GTPases is in understanding how they are inactivated during the absence or limitation of amino acids. A genome-wide screen performed in yeast identified two proteins, Npr2 and Npr3, which are part of a larger SEA complex, as major negative regulators of TORC1 function.[136] More recent studies now reveal how this complex inhibits TORC1 activity.[70, 130, 137–140] This complex, once again superbly conserved across eukaryotes, at its core comprises of Npr2, Npr3 and Iml1 in yeast (or Nprl2, Nprl3 and DEPDC5 in mammals), and is called the SEACIT complex (yeast) or GATOR1 (mammals). These three proteins function together as a complex, coming together during amino acid starvation, [130, 137] and Iml1 exhibits GAP activity against Gtr1, resulting in GTP hydrolysis to GDP, thereby inactivating the SEACIT complex.[137] Notably (in the context of general metabolic sensing), the assembly of the SEACIT complex is itself regulated by phosphorylation of Npr3, and this phosphorylation is reversed by the protein phosphatase PP2A when cytosolic methionine/*S*-adenosyl methionine levels are high (and thereby methylating the C-terminus of PP2A, altering substrate specificity).[70] Both SEACIT and GATOR1 are parts of larger, functionally analogous but not homologous complexes in yeast and mammals, called SEACAT and GATOR2.[130, 137, 140] These complexes appear to be positive regulators of TORC1, by inhibiting SEACIT/GATOR1, though how this happens is unknown and remains an area of intense investigation. Many recent studies now show that the SEACIT/GATOR1 complex are critical for normal TORC1 function.[109, 130, 137, 141, 142]

5.4 Amino Acid Sensing from the Cytoplasm to the Lysosome

As the example of the LeuRS shows, it is also clear that free amino acids are sensed in the cytoplasm, and somehow transmit that information to the lysosome/vacuole. Some recent discoveries dramatically expand this aspect of amino acid sensing. Several simultaneous, independent studies have identified the Sestrin group of proteins (Sestrin 1–3) as negative regulators of TORC1 activity, upstream of the Rag GTPases.[143–146] These proteins, present only in metazoans (and not yeast), seem to be specifically inactivated directly by leucine.[147, 148] These studies suggest slightly different models on how Sestrins work. One study suggests that Sestrins bind to the RagA/B GTPases, and prevent GDP dissociation from them, keeping them in an inactive state.[143] The others suggest that Sestrins associate with GATOR2 during amino acid depletion, and thereby reduce the inhibition of the TORC1 inhibitor, GATOR1.[144–146] This series of inhibitions effectively inhibit the TORC1. Thus, the modulation of TORC1 activity by Sestrins seem to have dual mechanisms, through regulating RagA/B and by acting through the GATOR2 complex to regulate the Rag heterodimer activity. Regardless of their precise mechanisms, the Sestrin proteins seem to be physiologically very important for TORC1 function.[143, 146, 149]

Another, vertebrate specific protein, called CASTOR, has recently been identified as a specific amino acid sensor for arginine, directly binding arginine to be activated.[125] Their proposed mechanism of action is somewhat analogous to that of the Sestrins and leucine mediated regulation. During arginine starvation, dimers of CASTOR (1 and/or 2) are thought to bind the GATOR2 complex, thereby inhibiting it and TORC1 activity.[125] Although both Sestrins and CASTOR work in the cytoplasm, eventually they come together to regulate RAG function and TORC1 activity associated with the lysosome, highlighting the central role of the lysosome in amino acid sensing and TORC1 function.

5.5 Back to the Lysosome/Vacuole, and the Vacuolar ATPase

Thus far, we have emphasized the role of the Rag GTPases, in amino acid dependent activation of TORC1. However, all critical players in the amino acid dependent activation of TORC1 appears to be proteins integral to lysosomal/vacuolar function, particularly the v-ATPase, as well as other lysosome membrane-resident transporters.[86, 150] A specific glutamine/arginine transporter, SLC38A9 was recently identified as a key transducer of amino acid availability to the Rag

GTPases, [116, 126, 151] and this appears to act in conjunction with the V-ATPase. The role of the v-ATPase itself in amino acid sensing and activation of TORC1 emerged clearly from an RNAi screen in *Drosophila* cells, where it was found that v-ATPase catalytic activity was critical for proper Rag GTPase functioning.[116] This v-ATPase activity connected the Ragulator with Rag GTPase activity, and mTORC1 recruitment at the lysosome upon amino acid stimulus, [116, 121, 130, 131, 152] and the assembly of the v-ATPase itself appears to be amino acid dependent.[152] Interestingly, there appear to be conditions where amino acids can also activate the TORC1 independent of the Rag GTPases, where glutamine can be sensed by TORC1 in a lysosome and v-ATPase-dependent manner, through the vesicle trafficking regulator Arf1.[121] All of these data suggest that the roles played by the v-ATPase in amino acid sensing and TORC1 function are likely to be multi-faceted, highly regulated and nuanced, and the v-ATPase is likely to emerge as a major hub of amino acid signaling. Finally, a number of studies particularly in yeast have revealed that several vacuolar/lysosomal proteins involved in vacuolar protein sorting, general vacuolar function, and vescicle trafficking are critical for Rag GTPase function, and TORC1 activity.[117, 153–155]

Collectively, amino acid signaling through TORC1 has thrown up several surprises. It appears likely that there is not a single amino acid "sensor", but multiple sensing hubs through which TORC1 is assembled (summarized in Fig. 2). All of these hubs lead to the surface of the lysosome/vacuole where amino acid sensing and signaling occur, TORC1 is properly assembled, and regulated in an amino acid dependent manner. It is not surprising, therefore, that the lysosome is emerging as this exciting hub of metabolism and signaling.[86, 156] It is likely that the coming years will solve many of the mysteries of amino acid sensing and signaling, unveil new amino acid sensors, and bring the lysosome/vacuole to the center stage as a major metabolic and signaling hub (Fig. 2).

5.6 Autophagy and Metabolic Outputs of TORC1

The process of autophagy is also intimately linked to lysosome/vacuolar function, amino acid sensing and the TORC1. Upon various nutrient starvations, but especially nitrogen/amino acid starvation, cells enter autophagy.[157–159] The distinguishing feature of autophagy is the formation of a double-membrane structure, the autophagosome, upon autophagy induction by starvation. During autophagy, cytoplasmic, mitochondrial and peroxisomal proteins and organelles are confined within the autophagosome, which fuses with the lysosome (or vacuole) and enables the degradation of the inside contents.[157–159] This process is highly regulated, and the activation of autophagy depends on TORC1 activity.[157, 158, 160] During amino acid replete conditions, TORC1 is active, and phosphorylates and inactivates key autophagy initiators, while when amino acids are scarce and TORC1 is inactive, these initiators of autophagy are activated and allow the formation of the autophagosome. Collectively, this interplay between the autophagy machinery and TORC1 allow the cell to maintain amino acid homeostasis. While a detailed description of the regulation of autophagy is beyond the scope of this review, these studies have been extensively described in several excellent reviews.[158, 159, 161, 162]

Finally, a short note on the nature of the actual metabolic outputs (as opposed to the signaling, translational and growth outputs leading to metabolic changes) regulated directly by TORC1, which we now better understand. Many of the metabolic outputs of TORC1 can be directly connected to amino acid metabolism. In particular, TORC1 is a potent activator of both purine and pyrimidine nucleotide synthesis, and this appears to be conserved from yeast to mammals.[122, 163–165] In addition, particularly through branched chain amino acids, TORC1 appears to control flux through the TCA cycle.[123, 166–168] TORC1 is also emerging as a key regulator of lipid metabolism (reviewed in [112]). Understanding mechanisms by which TORC1 directly regulates such key metabolic outputs will be a major area of interest in the coming years.

6 Many Questions for the Future

While the field of metabolic sensing and signaling has recently expanded dramatically, we are left with many unanswered, fundamental questions. The identities of multiple amino acid sensors are yet to be determined, and how the v-ATPase and the vacuole regulate amino acid sensing and homeostasis remain unanswered. Several central and intermediate metabolites appear to be functioning as signaling molecules, and both the mechanism by which they function, as well as their physiological significance, remain unexplored. We also have only a nascent understanding of intracellular metabolite pools, and how such pools are maintained. There are no effective

in vivo sensors for most metabolites, such as acetyl-CoA, *S*-adenosyl methionine, intermediates from the TCA cycle and more, and metabolite pools are highly dynamic, making it all the more necessary to develop effective in vivo sensors to enable live imaging of these molecules. Many questions remain on how metabolic sensing and signaling integrate with the process of protein translation. Thus, this field appears poised for dramatic expansion in the coming years.

Acknowledgements

SL would like to thank Adhish Walvekar and Ritu Gupta for assistance with the figures. An intermediate fellowship from the Wellcome Trust-DBT India Alliance (IA/I/14/2/50123) and funding from the DBT (BT/PR13446/COE/34/30/2015) to SL support the SL lab.

Received: 23 October 2016. Accepted: 14 November 2016
Published online: 22 March 2017

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