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# A brief history of TOR

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## Abstract

The TOR (target of rapamycin) serine/threonine kinases are fascinating in that they influence many different aspects of eukaryote physiology including processes often dysregulated in disease. Beginning with the initial characterization of rapamycin as an antifungal agent, studies with yeast have contributed greatly to our understanding of the molecular pathways in which TORs operate. Recently, building on advances in quantitative MS, the rapamycin-dependent phosphoproteome in the budding yeast *Saccharomyces cerevisiae* was elucidated. These studies emphasize the central importance of TOR and highlight its many previously unrecognized functions. One of these, the regulation of intermediary metabolism, is discussed.

## EN APXH ἡν ραπαμυκίνη (In the beginning was rapamycin; John 1:1 with modification)

Ancient civilizations knew well the medical value of natural products: indigenous South Americans, for example, used cinchona bark to treat fevers for many centuries while records for analgesic preparations from willow and other salicylate-rich plants date back to 3000 BCE. Building on such observations, systematic 'bioprospecting' expeditions, often to remote corners of the globe, were initiated in modern times to try to exploit Nature's pharmacopoeia. Indeed, the history of TOR (target of rapamycin) started with the efforts of a Canadian expedition, in the 1960s, to Easter Island (Rapa Nui in the native language) to gather plant and soil samples for subsequent analyses.

Importantly, one of these soil samples contained the bacterium *Streptomyces hygroscopicus* that was found to produce a secondary metabolite, now known as rapamycin, with potent antifungal activity [1,2]. Not long after its initial characterization as an antifungal agent, rapamycin was found to possess cytostatic activity not only against lower eukaryotes but also against mammalian cells, particularly immune cells and human tumour cells xenografted into rodents [3,4]. These impressive characteristics of this novel macrocyclic lactone led to the question: what is the target of rapamycin?

## The Big Bang

Although most appreciated at the time for its anti-cancer and immunosuppressive potential, it was the antifungal property of rapamycin that led to the discovery of its molecular target [5]. This was achieved using a simple, yet

elegant, selection of spontaneous mutants of the budding yeast *Saccharomyces cerevisiae* for the ability to form colonies on plates containing a cytostatic concentration of rapamycin. Three classes of mutants were recovered in this selection with the most populous class demonstrating recessive resistance to rapamycin. These mutants harboured defects in the *FPR1* gene, which encodes a non-essential proline isomerase that is an obligate cofactor required for rapamycin toxicity. The two other loci yielded dominant resistance to rapamycin and they were named *TOR1* and *TOR2*. Cloning and sequencing of these genes demonstrated that they encode huge paralogous (a quirk of yeast) kinases that resemble phosphatidylinositol kinases [6,7]. Today we know that TORs are conserved in nearly all eukaryotes (metazoans encode only a single *TOR* gene) and that they function not as lipid kinases, but rather as serine/threonine protein kinases [8,9].

## Flavours and colours

Biochemical purification of Tor1 and Tor2 from yeast demonstrated that these proteins function in at least two distinct multiprotein complexes named TORC (TOR complex) 1 and TORC2 [10,11]. Each complex appears to be conserved in higher eukaryotes [10,12–15] and each appears to perform one or more essential functions [10,16–19]. Table 1 provides a summary of the proteins that make up TORC1 and TORC2 in budding yeast and in mammals (humans). Importantly, rapamycin only binds to TOR in TORC1, and thus only the kinase activity of TORC1 is inhibited following acute treatment with rapamycin. As indicated in Table 1, not all components of these complexes are stably associated with the core complex, suggesting that these two 'flavours' of TOR each also come in different 'colours'.

## Space, time

The two TORCs influence many aspects of eukaryote physiology. Much of this influence, it seems, is a direct consequence of the ability of the TORCs to regulate growth.

**Key words:** antifungal agent, intermediary metabolism, rapamycin, target of rapamycin (TOR), target of rapamycin complex (TORC), yeast.

**Abbreviations used:** Fba1, fructose-1,6-bisphosphate aldolase; Gdh2, glutamate dehydrogenase; PRPP, phosphoribosyl pyrophosphate; PRPS, 5-phosphoribosyl-1(α)-pyrophosphate synthetase; TOR, target of rapamycin; TORC, TOR complex.

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**Table 1 | Summary of TOR and TOR-associated proteins found in the two TORCs**

For each TORC, the yeast and mammalian (human) orthologues are given in the same row [8,20–27]. Underlined proteins are found associated with the complex only under specific conditions. Multiple isoforms of mSin1 additionally define distinct forms of mTORC2 [28]. Raptor, regulatory associated protein of mTOR; rictor, rapamycin-insensitive companion of mTOR.

TORC1		TORC2	
Humans	<i>S. cerevisiae</i>	Humans	<i>S. cerevisiae</i>
mTOR	Tor1 or Tor2	mTOR	Tor2
mLst8	Lst8	mLst8	Lst8
Raptor	Kog1	Rictor	Avo3
<u>PRAS40</u>	–	–	Avo2
–	Tco89	mSin1	Avo1
<u>Deptor</u>	lml1?	<u>Deptor</u>	lml1?
		Protor1/2	Bit61/Bit2

Growth, i.e. the accumulation of mass, must be regulated in both time and space and there are now numerous examples of how the two TORCs operate in this regard.

The initial observation that TOR regulates growth was made in yeast with the demonstration that rapamycin-sensitive TORC1 promotes protein synthesis when nutrient conditions are favourable for yeast growth [29]. However, the ability of TORC1 to couple nutrient cues to the growth machinery is limited neither to yeast nor to single cells. TORC1 in the *Drosophila* fat body responds to amino acid cues to alter the growth of the entire larva [30]. In honey bees too, hyperactivation of TORC1 in larvae fed on royal jelly is necessary for the subsequent development of these larvae into queens rather than workers [31]. TORC1 also regulates growth at a ‘sub-organismal’ level. Load-bearing exercise induces a TORC1-dependent increase in muscle mass in vertebrates [32], and elegant studies in sea slugs and crayfish have demonstrated that TORC1-dependent *de novo* neuronal protein synthesis is required for long-term facilitation (long-term memory formation) [33]. Consistent with this later observation, recent evidence suggests that TORC1 plays a role in additional complex cognitive functions such as pregnancy-induced food preferences in fruitflies [34]. Furthermore, preliminary data suggest that hyperactivation of TORC1 in the prefrontal cortex could be an efficacious way of treating human depression [35]. In contrast, too much TORC1 activity, as seen in patients that have inherited/acquired a defective copy of any number of tumour suppressors that normally function to antagonize mammalian TORC1 activity, results in the development of hamartomas. Hamartomas are benign tumours of multiple tissues characterized by the presence of huge dysmorphic cells [36]. Indeed, given the number of oncoproteins and tumour suppressors that respectively activate and antagonize its activity, mammalian TORC1 is thought to be hyperactive in a majority of cancers [8]. Lastly,

although mechanistic details are still unclear, reduced TORC1 activity increases lifespan in yeast, nematode worms, fruitflies and rodents [37].

Lacking a rapamycin-equivalent tool with which to interrogate its function, understanding of the pathways downstream of TORC2 has lagged in comparison with TORC1. Genetic studies have suggested that TORC2 plays a prominent role in regulating spatial aspects of cell growth (reviewed in [38]). For example, depletion of TORC2 in *S. cerevisiae* and *Dictyostelium discoideum* or knockdown of mammalian TORC2 components leads to defects in actin organization. Furthermore, in slime moulds and human tissue culture cells, TORC2 regulates migratory responses and organelle distribution [39–41].

Additional functions of TORC2 have also been described. In *S. cerevisiae* and *Caenorhabditis elegans*, TORC2 regulates lipid synthesis [42–44], while in *Drosophila melanogaster* it controls the dendritic tiling of sensory neurons [45]. In the fission yeast, *Schizosaccharomyces pombe*, TORC2 influences both stress responses as well as cell-cycle progression [46].

## Black hole

Although TORC1, by coupling growth decisions to environmental cues, and TORC2, by directing mass deposition to discreet loci, generally appear to regulate temporal and spatial aspects of cell growth, it is far from clear, at the molecular level, how the many readouts now ascribed to these two complexes are controlled. Indeed, very few direct substrates of the TORCs are known.

## Beyond the event horizon

From the discussion above, it is hopefully clear that a more complete understanding of the molecular pathways downstream of the two TORCs is not only academically interesting, but also potentially clinically interesting. To this end, our group, together with Ruedi Aebersold’s group, has recently employed a novel MS approach to ascertain the rapamycin-sensitive (and thus presumably the TORC1-dependent) phosphoproteome in budding yeast [47]. Specifically, we employed a novel label-free yet quantitative MS approach to define the rapamycin-sensitive phosphoproteome in an unbiased manner. In a complementary study, the arguably more standard SILAC (stable isotope labelling with amino acids in cell culture) MS approach was similarly employed to characterize the rapamycin-sensitive phosphoproteome of budding yeast [48]. Although these MS approaches suffer from high false-negative rates, false-positive rates appear to be quite low and thus they nonetheless yield considerable insight into novel distal readouts downstream of TORC1. As many of the known readouts downstream of TORC1 have been reviewed recently [49,50], for the remainder of the present mini-review I focus on an underappreciated target of TORC1-dependent

signals suggested by these two phosphoproteome studies: the regulation of intermediary metabolism.

A role for yeast and mammalian TORC1 in the regulation of metabolism was first suggested by transcriptomics studies [51,52]. Subsequent transcript profiling experiments confirmed and extended these results, demonstrating that mammalian TORC1 activates a range of genes encoding enzymes involved in glycolysis, the pentose phosphate pathway and *de novo* lipid biosynthesis [53]. However, in addition to this regulation at the transcriptional level, the elucidation of the rapamycin-sensitive phosphoproteome of yeast suggests that many of these enzymes are directly regulated by TORC1 at the post-translational level.

### TORC1-dependent regulation of glucose and nitrogen intermediate metabolism

Glucose is the preferred carbon source for budding yeast, and glucose fermentation, rather than respiration, is the main metabolic pathway for both energy and carbon intermediates [54]. In this regard, yeast metabolism is rather similar to that of many tumour cells that likewise abandon oxidative phosphorylation in preference for 'aerobic glycolysis' known as the Warburg effect [55]. Arguably, the rate-limiting step of glycolysis is the unidirectional conversion of fructose 6-phosphate+ATP into fructose 1,6-bisphosphate+ADP catalysed by phosphofructokinase. In yeast, phosphofructokinase is an 835-kDa hetero-octamer made up of four  $\alpha$  (Pfk1) and four  $\beta$  (Pfk2) subunits in a  $\beta_2\alpha_4\beta_2$  configuration [56]. The activity of the holoenzyme is extensively regulated by allosteric interactions (up to 20 different compounds affect its activity [57]), with ATP inhibiting the enzyme and AMP and fructose 2,6-bisphosphate reversing the inhibition. Point mutations in either  $\alpha$  or  $\beta$  subunits that render phosphofructokinase insensitive to allosteric regulation suggest that regulation of the enzyme is important for growth under changing nutrient conditions [58]. Interestingly, both subunits appear to be differentially phosphorylated upon rapamycin treatment, with Pfk1 becoming dephosphorylated and Pfk2 becoming hyperphosphorylated [47]. Although these preliminary observations obtained in a high-throughput screen need still to be confirmed, they suggest the very interesting possibility that TORC1 signals directly impinge upon this key node of glycolysis.

Phosphofructokinase is not the only glycolytic enzyme apparently targeted by TORC1; phosphorylation of Fba1 (fructose-1,6-bisphosphate aldolase) appears also to be decreased upon rapamycin treatment [47]. Fba1 catalyses the conversion of fructose 1,6-bisphosphate into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate during glycolysis and the reverse reaction during gluconeogenesis [59]; it is tempting to speculate that the phosphorylation status of Fba1 may tip this balance.

PRPP (phosphoribosyl pyrophosphate) represents an important link between carbon and nitrogen metabolism. PRPP is a biosynthetic precursor of histidine and tryptophan,

and it is also required for the *de novo* and salvage pathways of purine, pyrimidine and pyridine ( $\text{NAD}^+$ ,  $\text{NADP}^+$ ) nucleotides. It is generated by the transfer of pyrophosphate from ATP to ribose 5-phosphate catalysed by PRPS [5-phosphoribosyl-1( $\alpha$ )-pyrophosphate synthetase]. PRPS is an important enzyme in the industrial production of riboflavin and, like other metabolic enzymes, is subject to allosteric regulation [60]. Mutations in human PRPS genes are associated with different hereditary disorders, including hyperuricaemia, mental retardation, developmental delay and other neurological pathologies [61–63]. In budding yeast, there are five related PRPS enzymes which function in heteromultimeric complexes [64]. One of these enzymes, Prs5, appears to be hyperphosphorylated upon rapamycin treatment [47], although the biological significance of this phosphorylation remains to be established.

Gdh2 (glutamate dehydrogenase) also appears to be hyperphosphorylated in cells following rapamycin treatment [48]. NAD-dependent Gdh2 degrades glutamate, yielding ammonia and oxaloacetate. The liberated ammonia can subsequently be reacted with a second molecule of glutamate to generate glutamine. In budding yeast, the amino group of glutamate and the amide group of glutamine are the source of nitrogen for biosynthesis of all other macromolecules [65]. Oxaloacetate is an important tricarboxylic acid cycle intermediate as anapleurotic reactions can feed into the cycle at this juncture. Thus, in regulating glutamate, glutamine, ammonia and oxaloacetate levels, Gdh2 plays a role of central importance in nitrogen metabolism. Consistent with such a central role, the activity of Gdh2 has been proposed to be regulated by (potentially TORC1-dependent [66]) phosphorylation which appears to inactivate the enzyme [67].

### TORC1-dependent regulation of nucleotide and amino acid synthesis

Amd1 is a tetrameric enzyme that catalyses the deamination of AMP to form IMP and ammonia. Upon transition from respiration to fermentation (for example, upon the addition of glucose to a yeast culture respiring a non-fermentable carbon source) there is a dramatic fall in ATP levels. Owing to the action of adenylate kinase activity, this would normally lead to an increase in AMP levels that would, through allosteric interactions, have a significant, and in this case inappropriate, effect on subsequent glycolytic steps. To circumvent this, during this transition, AMP is rapidly converted into IMP by the action of Amd1 [68]. The observation that Amd1 is dephosphorylated upon rapamycin treatment [47,48] might suggest that, in response to environmental cues, TORC1 regulates Amd1 activity to allow cells to manage AMP levels. Such a regulation may also play a role in humans, as individuals harbouring defective alleles of AMP deaminase display deficiencies in physical performance [69].

The uridine kinase Urk1p is also dephosphorylated upon rapamycin treatment [48]. Urk1 phosphorylates uridine into UMP and cytidine/deoxycytidine into CMP/dCMP in the pyrimidine (deoxy)ribonucleotide salvage pathway [70,71]. These two pathways provide pyrimidines required for nucleic

acid synthesis, amino acid synthesis and energy [71]. The physiological significance of this phosphorylation remains to be determined.

Unlike mammalian cells, yeast cells can synthesize tetrahydrofolate (vitamin B<sub>9</sub>) and subsequent folate derivatives which are essential cofactors in one-carbon transfer reactions, including the synthesis of methionine and purines. The *Fol1* gene of yeast has dihydropteroate synthetase, dihydro-6-hydroxymethylpterin pyrophosphokinase and dihydroneopterin aldolase activities and thus catalyses three separate steps in folate synthesis [72]. Fol1 is dephosphorylated upon rapamycin treatment [47], raising the possibility that its activity is regulated by TORC1. Interestingly, Fol1 is found in the mitochondria [72], whereas TORC1 localizes predominantly to the vacuolar membrane [49]. It will be interesting to see if, how and why TORC1 signals cross the mitochondrial membranes to influence Fol1 phosphorylation.

Lastly, several additional enzymes involved in the biosynthesis of amino acids are differentially phosphorylated upon rapamycin treatment and thus also appear to be potentially regulated by TORC1 signals [47,48]: Ser33, required for serine and glycine synthesis; Met2 and Met12, required for methionine synthesis; Hom3, required for methionine and threonine synthesis; and Lys12, required for lysine synthesis.

### TORC1-dependent regulation of metabolic reserves

Accumulation of carbohydrates and lipids is an important response to starvation [73]. It is perhaps not surprising therefore that TORC1 inhibition with rapamycin, which in many regards causes cells to behave as if they were starved of nutrients (particularly nitrogen) [49], alters the phosphorylation of enzymes involved in mobilization of metabolic reserves.

Sterols are essential lipids for eukaryote cells. Free sterols are synthesized in the endoplasmic reticulum but are concentrated in the plasma membrane. Steryl esters accumulate in intracellular lipid bodies and serve as a storage form of sterols and fatty acids. Three membrane-anchored lipases have recently been described to be necessary to hydrolyse steryl esters and thus mobilize free sterols [74]. One of these, Tgl1, is dephosphorylated upon rapamycin treatment [48], suggesting that TORC1 plays a role in sterol mobilization.

Tgl5 becomes hyperphosphorylated upon rapamycin treatment [47], suggesting that TORC1 also plays a role in the mobilization of TAGs (triacylglycerols). Interestingly, Tgl5 has both a TAG lipase domain as well as a lysophosphatidic acid acyltransferase domain. Thus this enzyme can function in both anabolic and catabolic pathways [75]; perhaps TORC1-dependent phosphorylation favours one over the other.

In addition to lipid reserves, carbohydrate reserves are also known to be influenced by TORC1 activity. For example, inhibition of TORC1 is well known to result in glycogen accumulation [49]. In budding yeast, glycogen, a branched

polysaccharide of high molecular mass, is catabolized to glucose 1-phosphate by the glycogen phosphorylase Gph1. *gph1*-null cells accumulate glycogen, suggesting that the increase in Gph1 phosphorylation observed upon rapamycin treatment [48] may lead to inactivation of the enzyme.

In yeast, the disaccharide trehalose functions not only as a carbohydrate reserve, but also probably as a molecular chaperone required for surviving thermal, osmotic, oxidative and ethanol stress [76]. Trehalose is synthesized from uridine-5'-diphosphoglucose and glucose 6-phosphate by the trehalose-6-phosphate synthase/phosphatase complex. This complex is composed of Tps1, the synthase subunit, Tps2, the phosphatase subunit and two redundant regulatory subunits Tps3 and its paralogue Tsl1. Both regulatory subunits are hyperphosphorylated upon rapamycin treatment [48], which may help begin to explain how yeast cells accumulate trehalose following TORC1 inhibition [77].

### Metabolism: the final frontier

With the advent of ultrahigh-throughput sequencing technologies, genomic and transcriptomic studies have now become routine. Recent MS advances have also made proteomic and lipidomic studies much more feasible. In contrast, identifying the hundreds of distinct small-molecule metabolites in a given cell and quantifying the flux of their synthesis still remains rather challenging [78]. Many observations, however, suggest that it is critically important that researchers are able to acquire high-quality metabolomics data. For example, for more than 50 years it has been known, but not understood, that tumour cells display an altered metabolism, typically an increase in aerobic glycolysis [79]. On the basis of this observation, the idea of targeting tumour cell energy metabolism, the so-called 'metabolic therapy', as a cancer therapy has been advanced [55]. More recently, nutrient excess coupled with reduced physical activity in Western societies has led to a dramatic increase in the metabolic syndrome, diabetes and cancer [80]. One might hope that metabolic profiling of such patients will enable better diagnoses and treatments. To this end, fluorodeoxyglucose-based positron emission tomography is already used in the clinic to monitor tumour response to chemotherapeutics [81]. In the meantime, as aberrant hyperactivation of mammalian TORC1 appears to be a common molecular event in hamartomatous tumour syndromes, cancers and obesity, the elucidation of the metabolic targets of TORC1 is of particular interest. Furthermore, given that core metabolic pathways are robustly conserved in eukaryotes and that tumour cell energy metabolism has been suggested to share several common features with yeast metabolism [55], studies in budding yeast are well positioned to make significant contributions in this regard.

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