## COMMENTARY

# Nutrient-responsive mTOR signalling grows on *Sterile* ground

Simon J. COOK*[1] and Simon J. MORLEY†[1]

*Laboratory of Molecular Signalling, The Babraham Institute, Babraham Research Campus, Cambridge, CB22 3AT, U.K., and †Department of Biochemistry, School of Life Sciences, University of Sussex, Falmer, Brighton BN1 9QG, U.K.

The control of cell growth, that is cell size, is largely controlled by mTOR (the mammalian target of rapamycin), a large serine/threonine protein kinase that regulates ribosome biogenesis and protein translation. mTOR activity is regulated both by the availability of growth factors, such as insulin/IGF-1 (insulin-like growth factor 1), and by nutrients, notably the supply of certain key amino acids. The last few years have seen a remarkable increase in our understanding of the canonical, growth factor-regulated pathway for mTOR activation, which is mediated by the class I PI3Ks (phosphoinositide 3-kinases), PKB (protein kinase B), TSC1/2 (the tuberous sclerosis complex) and the small GTPase, Rheb. However, the nutrient-responsive input into mTOR is important in its own right and is also required for maximal activation of mTOR signalling by growth factors. Despite this, the details of the nutrient-responsive signalling pathway(s) controlling mTOR have remained elusive, although recent studies have suggested a role for the class III PI3K hVps34. In this issue of the *Biochemical Journal*, Findlay et al. demonstrate that the protein kinase MAP4K3 [mitogen-activated protein kinase kinase kinase kinase-3, a Ste20 family protein kinase also known as GLK (germinal centre-like kinase)] is a new component of the nutrient-responsive pathway. MAP4K3 activity is stimulated by administration of amino acids, but not growth factors, and this is insensitive to rapamycin, most likely placing MAP4K3 upstream of mTOR. Indeed, MAP4K3 is required for phosphorylation of known mTOR targets such as S6K1 (S6 kinase 1), and overexpression of MAP4K3 promotes the rapamycin-sensitive phosphorylation of these same targets. Finally, knockdown of MAP4K3 levels causes a decrease in cell size. The results suggest that MAP4K3 is a new component in the nutrient-responsive pathway for mTOR activation and reveal a completely new function for MAP4K3 in promoting cell growth. Given that mTOR activity is frequently deregulated in cancer, there is much interest in new strategies for inhibition of this pathway. In this context, MAP4K3 looks like an attractive drug target since inhibitors of this enzyme should switch off mTOR, thereby inhibiting cell growth and proliferation, and promoting apoptosis.

Key words: amino acid signalling, cell growth, MAP4K3, mTOR, Ste20.

mTOR (the mammalian target of rapamycin) is a protein kinase that regulates multiple cellular signalling pathways and functions, including protein synthesis in response to growth factors, nutrients and amino acids (especially leucine and arginine). As such, mTOR plays a central role in regulating and integrating signals controlling cell growth, proliferation, apoptosis and autophagy [1]. Recent studies indicate that mTOR exists in two functional complexes, mTORC1 and mTORC2 [2], both associated with mLST8. However, Raptor (the regulatory associated protein of mTOR) and Rictor/mAVO3 (the rapamycin-insensitive companion of mTOR) are exclusively associated with mTORC1 and mTORC2 respectively, with only the former complex sensitive to acute treatments with rapamycin [3,4]. mTORC1 itself positively requires association with Rheb, a small GTPase protein localized to intracellular membranes, for activity and is inhibited by the TSC1/2 (the tuberous sclerosis complex). In a poorly defined pathway, this protein complex, which acts a GAP (GTPase-activating protein) for Rheb, inhibits mTORC1 activity by inactivating Rheb, with Rheb-GDP unable to support the mTORC1 complex (reviewed in [1]).

The generally accepted view is that mTORC1 recruits p70S6K1 (p70 S6 kinase 1) and 4E-BP1 (4E-binding protein 1) to the complex via Raptor using a specific TOS (Tor signalling) motif; mTOR then phosphorylates them at specific sites, and thereby regulates protein synthesis rates and cell growth (reviewed in [1,5]). Removal of amino acids (or simply leucine) causes a dramatic decrease in the phosphorylation of these mTORC1 targets, suggesting regulation at the level of mTOR signalling. However, although mTORC1 signalling in response to growth factors is sensitive to wortmannin [6], there is still some contention as to whether amino acid deficiency does [7] or does not [8] affect Rheb-GTP levels. These studies infer that amino acids regulate the binding of Rheb-GTP to mTOR, or suggest another mode of regulation of mTOR under these conditions [6,8]. The latter appears to be mediated via a parallel pathway using hVps34, which localizes to the endosomes [9], binds to mTORC1 and whose lipid kinase activity is sensitive to amino acid levels [6,9,10].

In this issue of the *Biochemical Journal*, Findlay et al. [11] have now identified an entirely new component on the amino acid-sensitive signalling pathway to mTOR. The authors used an RNAi (RNA interference) screen in *Drosophila* S2 cells to identify CG7097, a protein kinase that was required for phosphorylation of dS6K, the *Drosophila* orthologue of S6K1; the screen involved suppression of dTsc1, to activate dS6K, accompanied by co-suppression of approximately 200 *Drosophila* protein kinases. Homology searching revealed that the closest human orthologue of CG7097 was MAP4K3 (mitogen-activated protein kinase kinase kinase kinase-3, also known as GLK for germinal centre-like kinase) a member of the MAP4Ks related to the *Ste20* (*Sterile20*) protein kinase involved in the yeast mating pheromone response pathway. Knockdown of MAP4K3 in human HeLa cells caused strong inhibition of the increase in S6K1 and S6 phosphorylation observed upon re-addition of amino acids to depleted cells, and this was independent of TSC1 and TSC2,

[1] Correspondence may be addressed to either author (email simon.cook@bbsrc.ac.uk or s.j.morley@sussex.ac.uk).

which operate on the growth-factor-regulated pathway. In addition, simple overexpression of wild-type MAP4K3 (but not a catalytically inactive mutant) was sufficient to stimulate S6K1 activity, phosphorylation of S6 and phosphorylation of 4E-BP1, another mTOR target, but not phosphorylation of PKB (protein kinase B). These events were sensitive to rapamycin, suggesting that MAP4K3 signals via mTOR to activate S6K1 and 4E-BP1. The authors were also able to show that re-addition of amino acids to cells caused a rapid increase in MAP4K3 activity; this is an important observation, because a trivial explanation of the RNAi knockdown experiments was that MAP4K3 was simply required for the expression of a component on the nutrient-sensing pathway but was not actually part of the pathway itself. The activation of MAP4K3 was not observed in response to insulin, preceded the phosphorylation of S6K1, as might be expected for an upstream kinase, and was independent of rapamycin, indicating that it was upstream of (or parallel to) mTOR. Finally, to underpin the importance of their observations, the authors were able to show that RNAi-dependent knockdown of MAP4K3 caused a decrease in cell size that was comparable with that seen upon inhibition of Rheb or treatment with rapamycin, showing that MAP4K3 is important in the maintenance of cell size.

These results are important because they describe a new component involved in the amino acid-sensing pathway for mTOR activation. However, as is so often the case, the study also raises a lot of questions. First, what is the function of MAP4K3 in the amino-acid-sensing pathway to mTOR? Comparisons with other Ste20-like kinases provide relatively little clear information to guide us. Phylogenetic analysis reveals that there are two PAK-like and eight GCK-like Ste20 subfamilies, and MAP4K3 lies within the GCK-1 subfamily where its closest relatives are GCK and HPK1 (haematopoietic progenitor kinase 1) [12]. By virtue of it being a MAP4K, it is tempting to place MAP4K3 quite high up on a hierarchical signalling pathway; indeed, the rapid activation of MAP4K3 following addition of amino acids [11] provides circumstantial evidence for this. Support for a proximal role in signalling also comes from the literature, where MAP4K3 (as GLK) was activated in response to TNF$\alpha$ (tumour necrosis factor $\alpha$) stimulation and, in common with GCK and HPK1, could activate the stress-activated protein kinase JNK (c-Jun N-terminal kinase) via a MAP4K3/MEKK1/MKK4/JNK cascade upon overexpression [13]. However, there are no obvious links from JNK to activation of mTOR targets, and studies in *Drosophila* indicate that JNK actually antagonizes insulin-dependent signalling for cell growth [14].

Another obvious and pressing question arising from this study is the relationship between MAP4K3 and hVps34 [10], and MAP4K3 and mTORC1. Specifically, is MAP4K3 upstream, downstream or associated with Vps34? Is MAP4K3 recruited directly to mTORC1 or recruited in a complex with hVps34, in a similar manner to that described for hVps34/hVps15 [6]? Does MAP4K3 phosphorylate mTOR directly on Ser-2448 and modulate its activity in a manner analogous to that of PKB [15]? Clearly, a priority is to examine the impact of inhibiting Vps34 expression and/or activity on amino-acid-dependent activation of MAP4K3. This should be a relatively straightforward undertaking, since Findlay et al. show that MAP4K3 undergoes a robust 8- or 9-fold activation upon re-addition of amino acids, providing a good window in which to observe inhibition [11]. A simple starting place would be the non-selective PI3K inhibitor, wortmannin. Since there is no evidence that class I or II PI3Ks are involved in amino acid signalling, sensitivity to wortmannin could be used to implicate a PI3K of some sort upstream of MAP4K3, and comparisons between wortmannin and some of the novel class I-specific PI3K inhibitors that are now available [16] might be informative. Alternatively, it could be determined whether MAP4K3 activity is sensitive to overexpression of GFP (green fluorescent protein)–FYVE, as described for hVps34. Ultimately, such results will require molecular confirmation by Vps34-specific RNAi as reported previously [6,10].

At first sight, the involvement of a Ste20 kinase in the mTOR pathway seems to come completely out of left field and little, if anything, is known about direct links between Vps34 and Ste20 kinases. However, there are hints in the literature which start to draw Vps34, MAP4K3 and mTOR together. Analysis of the primary amino acid sequence of MAP4K3 reveals the N-terminal kinase domain and the C-terminal citron homology (CNH) domain to be highly conserved from the worm and fly to man. The CNH domain is a poorly defined protein–protein interaction domain that is found at the C-terminus of the GCK-I and GCKIV sub-families of Ste20 kinases. However, the homology between the CNH domains of the class I and class IV GCKs is rather low, and the domain appears to serve different functions in each class [12]. For example, in the class I GCKs the CNH domain appears to bind to small GTPases; MAP4K2 (the original GCK) binds to Rab8 [17] and MAP4K4 (HPK1) binds to Rap2 [18], and this is also consistent with suggestions that the CNH domain is found in Rho exchange factors and some Rac or Rho targets [19]. In contrast, in the class IV GCKs the CNH domain may bind to MEKK1 [12]. The mid-section of MAP4K3 is a poorly conserved stretch of low complexity but does include two proline-rich segments that are conserved in mammals. One of these, PPRPPPPR, has been show to interact with the SH3 domain of endophilin I [20] suggesting a link between MAP4K3 and endocytic vesicle formation. Interestingly, the closely related MAP4K2 is also linked with membrane trafficking events by virtue of its ability to bind to the GTP-bound form of Rab8, a small GTPase involved in vesicular transport, via the CNH domain [17]. Since components of the endocytosis and autophagy machinery are increasingly recognized as regulators or effectors of mTOR [9], the placement of MAP4K3 on the same pathway as Vps34 in mTOR signalling no longer seems like quite such a leap.

mTOR serves to integrate nutrient and growth signals and so acts as a central controller of cellular and organism growth. It interacts with a variety of signalling pathways/systems to regulate cell size, proliferation, apoptosis and autophagy. Deregulation of mTOR signalling is increasingly linked to various disease states, so the identification of MAP4K3 as a new component of the pathway is an important fundamental advance that may have implications for future attempts at therapeutic intervention. For example, up to 50 % of all tumours may exhibit inappropriate activation of mTOR due to mutations in various components of the pathway including p110$\alpha$-PI3K, PKB, PTEN (phosphatase and tensin homologue deleted on chromosome 10), LKB1 and TSC2, with the result that inhibition of mTOR activity is an increasingly desirable therapeutic strategy for cancer [21]. In this context, MAP4K3 looks like an attractive new drug target, as any inhibitor of this enzyme might be expected to down-regulate mTOR activity, thereby inhibiting cell growth, proliferation and survival. These results also point to potential new functions for the Ste20 family of protein kinases; a family with many members but, despite an extensive literature, relatively few rigorously defined biological functions.

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