**mTOR and cancer: many loops in one pathway**

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<td><a href="http://dx.doi.org/10.1016/j.ceb.2009.10.007">http://dx.doi.org/10.1016/j.ceb.2009.10.007</a></td>
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<td>Elsevier</td>
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mTOR and cancer: many loops in one pathway

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Summary
The mammalian target of rapamycin (mTOR) is a master regulator of cell growth and division that responds to a variety of stimuli, including nutrient, energy, and growth factors. In the last years, a significant number of pieces have been added to the puzzle of how mTOR coordinates and executes its functions. Extensive research on mTOR has also uncovered a complex network of regulatory loops that impact the therapeutic approaches aimed at targeting mTOR.

Introduction
Adequate cellular levels of energy and nutrients are a prerequisite for successful cell growth and division. Therefore, cells have acquired mechanisms to sense energy and nutrients before committing to grow and divide. In all eukaryotes, the target of rapamycin (TOR) protein is a master regulator that integrates the signals from nutrient and energy sensors with cell growth and proliferation, so as to ensure that they are triggered only during favorable conditions. The mammalian TOR (mTOR) also integrates growth factor signaling together with nutrients and energy as a mechanism to coordinate cell growth and proliferation in large numbers of cells within different organs. In addition to this, a network of regulatory loops affects the function of mTOR and impact on therapeutic approaches aimed at targeting mTOR, which will be discussed here.

The mTOR signaling pathway
mTOR forms two different protein complexes defined by the proteins to which it is bound, exerting different but related functions. The mTOR complex 1 (mTORC1) is defined by the presence of Raptor, mLST8/GβL, Deptor and Pras40 [1-7], whereas Rictor, GβL, Protor, Deptor and mSin form mTORC2 [8-12] (see Figure 1). In addition to their differential sensitivity to rapamycin, mTORC1 and 2 are activated in different ways and have distinct substrate specificity. mTORC1, which is sensitive to rapamycin, responds to energy, amino acids, growth factors and oxygen levels, whereas mTORC2 activation is ill-defined, but seems to be only by growth factors. Active mTORC1 phosphorylates, among other targets, S6K1 and 4EBP1, proteins involved in the regulation of translation initiation, protein synthesis and cell...
mass gain (see [13] for a comprehensive review). An overview of the mTOR complexes and signaling pathway is provided in Figure 1.

**The Rag - amino acid link**

The fact that amino acid-dependent activation of mTORC1 occurs independently of the Akt-TSC axis signaling has been known for years, but the identification of the molecular link between amino acids and mTORC1 has remained elusive. Recently, we and others showed that the Rag family of GTPases is required for mTORC1 activation by amino acids [14,15]. Rag proteins directly interact with Raptor in an amino acid-dependent manner, and this interaction leads to mTORC1 activation. Interestingly, the nucleotide-bound status of the Rag GTPases dictates its affinity to Raptor and the activation of mTORC1. This discovery shows a previously unknown regulatory mechanism of mTOR function that involves mTOR shuttling. In the presence of amino acids, Rag proteins mediate mTORC1 shuttle from disperse punctuate pattern in the cytoplasm to the endomembrane system of the cell, where signaling occurs [15]. According to this, appropriate localization and active PI3K-Akt-Rheb axis must coexist, explaining why both amino acids and growth factors are needed for activation of mTORC1. It is likely that Rag GTPases are not responsible for directly sensing amino acid levels; instead this information is probably communicated to the Rag proteins by additional mediators.

**Complex loops of regulation - all roads lead to mTOR**

mTOR, as a master regulator of a variety of inputs, is subjected to different mechanisms that tightly and coordinately regulate its activation. Many positive and feedback loops have been described lately and most likely other regulatory loops will be deciphered in the near future.

**The S6K1-PI3K inhibition**

A very important negative feedback involves the inhibition of PI3K by mTORC1, which occurs at many levels (see Figure 2A). Active S6K inhibits insulin receptor substrate 1 (IRS-1) by phosphorylating it at multiple sites, by inducing its degradation and by altering its localization, all of which ultimately dampen PI3K/Akt activation [16-19]. This loop is relevant in vivo and influences therapeutic responses based on mTOR inhibition (see below), and also plays a role in type 2 diabetes [20]. A functionally similar loop mediated by S6K1 is the inhibition of platelet-derived growth factor receptor (PDGFR), that also signals through PI3K [21]. Of note, the direct target of S6K1 that inhibits PDGFR activity is currently unknown. In addition, it has been recently shown that S6K1 is also responsible for inhibiting the activity of the ERK/MAPK pathway. This loop was empirically unmasked by therapeutic inhibition of mTORC1 in cancer patients, which correlated with an increase in ERK phosphorylation in vivo [22]. In spite of the fact that most research on mTORC1 is centered on S6K1, it is also conceivable that the effects on mTORC1 inhibition may not be solely through this target. These partially overlapping inhibitory loops have probably evolved as a mechanism to negatively control PI3K pathway after its activation, reflecting the necessity of putting a brake to excessive (acute or chronic) activation of the pathway.

**Intra-complex loops**

mTOR regulation involves a series of feedback loops triggered by mTORC1 and 2 components. Perhaps the most interesting loop where both mTOR complexes participate is the one involving mTOR regulation of Akt. It has been shown that mTORC2 is the kinase of Akt at the serine in position 473 [23]. This phosphorylation, together with the one at 308 performed by PDK1 upon PI3K activation, is needed for maximal Akt activity and conceptually locates mTOR both upstream and downstream Akt (Figure 2B).
Our group has recently described the role of Deptor in the regulation of both mTORC1 and 2 [5]. The functional relationship of Deptor and the mTORCs is not simple (Figure 2B). Deptor protein and mRNA levels are suppressed by mTORC1 and mTORC2 activation, and Deptor inhibits mTORC1 and 2 kinase activities \textit{in vitro} and in cell-based assays. Consequently, activation of mTORC1 and 2 leads to Deptor inhibition, leading to further mTOR activity and in contrast, high levels of Deptor inhibit mTOR activity. Importantly, inhibition of mTORC1 by Deptor indirectly activates Akt, by relieving the inhibitory loop on PI3K triggered by S6K1. Considering this, theoretically Deptor could have both tumor suppressive (by inhibition of mTORC1 and 2) and oncogenic roles (by the activation of Akt) (Figure 2B). Although more work and \textit{in vivo} data will help clarify this issue, transcriptional profiling of tumor cell lines showed that Deptor was overexpressed in multiple myeloma cells lines that show high Akt activity. In these cell lines, silencing of Deptor lead to inhibition of Akt and cell death, altogether suggesting that deregulated Deptor function may have oncogenic properties [5].

Recently, TSC, in addition to its role in the inhibition of mTORC1, was linked to mTORC2 function. TSC1/2 complex interacts with mTORC2 and positively regulates its kinase activity by an as yet undefined mechanism that seems to be independent of the inhibition of mTORC1 [24]. Although this finding requires further investigation, it locates TSC downstream and upstream of Akt. It also helps to explain the benign nature of tuberous sclerosis syndromes, because in TSC-deficient cells, in addition to the S6K1-PI3K-Akt feedback that inhibits Akt, its activity would be further diminished by reduced activation mTORC2. In addition, S6K1 was shown to regulate mTORC2 activity by direct phosphorylation of the mTORC2 component Rictor. This phosphorylation event does not regulate mTORC2 kinase activity \textit{in vitro} nor alters mTORC2 effects on most of its targets in cell-based experiments, but it negatively regulates phosphorylation of Akt at serine 473 [25]. These two new regulatory loops point towards a cellular mechanism that prevents simultaneous activation of mTORC1 and mTORC2, in addition to the already described role of S6K1 in putting a brake on excessive PI3K activation.

\textbf{p53 and mTORC1}

The tumor suppressor p53 is activated by different types of cellular stress, including DNA damage and oncogene activation. p53 mediates transactivation of a large number of genes involved in cell-cycle arrest and apoptosis. p53 also transactivates negative regulators of mTORC1, namely TSC2 and AMPKβ1 [26]. Besides, AMPK phosphorylates p53 at the serine in position 15, which leads to stabilization and favors the transactivation capacity of p53 [27]. Hence, p53 and AMPK positively regulate each other. Moreover, p53 upregulates the transcription of Sestrins 1 and 2, which bind to the ternary complex TSC1/2-AMPK, inducing phosphorylation and activation of TSC2 by AMPK [28]. In addition Pten was shown to interact with p53, leading to p53 stabilization [29]. Interestingly, Akt is a negative regulator of p53 activity by phosphorylating an E3 ubiquitin ligase, namely MDM2, which drives its translocation to the nucleus where it destabilizes p53 [30]. These cross regulations of AMPK, Pten and p53 oppose Akt activity (see Figure 2C) and constitute a network coordination of division and growth after sensing nutrient availability, DNA integrity and oncogenic activity.

\textbf{The impact of mTOR feedback loops in cancer}

\textbf{Rapamycin-based therapy}

Extensive basic research with rapamycin showed high specificity towards mTORC1 inhibition. This has encouraged a number of clinical trials using this compound as an anticancer drug, but in fact, rapamycin and its first generation analogs (temsirolimus and everolimus) have proven modest success in clinical trials, reflecting an incomplete understanding of mTOR functions. These small molecules are allosteric inhibitors of mTOR that block S6K1 phosphorylation,
but it has become evident that not all mTORC1 functions are blocked by rapamycin [31-35]. Rapamycin also stops the negative feedback loops emanating from S6K1 to PI3K signaling pathway, thus activating proliferative and prosurvival effectors, as Akt. This was shown to be relevant in many cancer cell lines and also in clinical samples [36], and may explain why rapamycin, although slowing proliferation in many cell lines, is a poor inducer of apoptosis. Altogether, this raises an important concern: if mTORC1 has rapamycin-resistant functions and rapamycin indirectly activates the PI3K signaling pathway, which is responsible for mTORC1 activation, then rapamycin could cause hyperactivation of those mTORC1-dependent functions not inhibited by rapamycin.

Akt is not the only target downstream PI3K activation but there is in vivo evidence that point towards Akt as perhaps the most important target affected by the feedback loop from S6K1, and contributing to the modest therapeutic success of rapamycin. TSC2-heterozygous mice have constitutive mTORC1 activity and, consequently, overactivation of the feedback loop that dampens Akt activity. Interestingly, double heterozygous mice for TSC2 and Pten have active Akt and accelerated tumorigenesis in some organs in comparison with single heterozygous mice [37,38]. Hay and colleagues [39] have shown that mTORC1 function downstream of PI3K-Akt is necessary for transformation and tumorigenesis in an experimental model of mammary and salivary tumors, favoring the hypothesis that rapamycin may be a good therapeutic strategy under conditions of Akt hyperactivation. However, Akt may drive tumorigenesis by regulating effectors different from mTORC1. In fact, Akt can regulate the Foxo proteins, cyclin D1, p27 and GSK3, all involved in cell cycle progression, and MDM2, caspase-9, IKKa and Bad, controlling the execution of apoptosis [30]. Only in those tumors where mTORC1 is the important downstream effector of the oncogenic activity of Akt, rapamycin would be a reasonable therapeutic strategy.

PI3K also has Akt-independent effectors, with potential clinical relevance, that would also be indirectly activated by rapamycin. For instance, high levels of PDK1 can sustain tumor growth in an Akt-independent manner, by hyperactivation of SGK3 [40]. SGK3 is part of the AGC family of proteins, that also includes Akt, and that share target specificity. Interestingly, SGK proteins are, as Akt, substrates of mTORC2 [41].

Although rapamycin is a highly selective mTORC1 inhibitor, this has been challenged by the fact that it can also block mTORC2 activity in a subset of cancer cell lines after prolonged treatment through a mechanism that may involve inhibition of mTORC2 assembly [42]. Moreover, high concentration of rapamycin was also shown to block mTORC2 activity [43]. However, whether such toxic high levels of rapamycin have a clinical applicability is unclear.

mTOR catalytic inhibitors

The development of a different class of mTOR inhibitors that blocks mTOR catalytic site (Torin [34], PP242 and PP30 [32], Ku-0063794 [33], and WAY-600, WYE-687 and WYE-354 [35]) was reported almost simultaneously by several groups. This approach presents an obvious advantage for therapeutics: by blocking mTOR, both mTORC1 and mTORC2 become inhibited. Consequently, all mTOR target activities are blocked, including Akt phosphorylation at position 473 [32-35]. The fact that mTORC2 is the kinase for Akt at Ser473 is now widely accepted and supported by genetic studies in mice, but it is important to determine to what extent the inhibition of this phosphorylation impairs Akt activity towards all of its targets, which may be differentially inhibited by abrogating Ser473 phosphorylation [9,44]. How phosphorylation at positions 308 and 473 cross-regulate Akt activity is particularly important in situations where S6K1 inhibition will boost PI3K activation (and, consequently, Akt phosphorylation at Thr308). Noteworthy, acute inhibition of mTORC2 by using these mTOR catalytic inhibitors suggests that phosphorylation at Ser473 is required for phosphorylation at
Thr308 and, consequently, for Akt activation. Further research should address how these compensatory loops would affect tumor responsiveness.

Toxicity is a possible caveat to consider if all mTOR functions are inhibited. However, work in mice suggests that blocking mTORC2 could be tolerated in vivo. In a murine model of Pten deficiency-driven prostate tumorigenesis, deletion of Rictor (i.e. mTORC2) partially impairs prostate adenocarcinomas development, but does not affect normal prostate gland function and architecture [45]. This indicates that mTORC2-specific inhibitors would be a good therapeutic strategy to be further investigated. In a similar experimental approach, Pandolfi and colleagues also showed that mTORC2 enhances prostate tumorigenesis, but ablation of mTOR in adult prostate does not affect normal prostate function [46], providing strong evidence that mTOR catalytic inhibitors could be well-tolerated in post-mitotic cells. Supporting this notion, WYE-354 inhibited human cancer cells growing as xenografts in nude mice with no detectable toxicity in the short term [35].

**Dual PI3K-mTOR inhibitors**

An alternative way to circumvent the activation of feedback loops that may preclude tumor responsiveness is the combined inhibition of PI3K together with mTOR. There are several small molecules that block PI3K and mTORC1 and 2, currently under intense investigation (reviewed in [47]). In cell lines and experimental tumors with hyperactive PI3K signaling, dual inhibition of PI3K and mTOR was effective [48], but failed in the presence of K-Ras hyperactivation. In Ras-driven tumorigenesis, concomitant inhibition of PI3K and mTOR, and also blocking downstream mediators of Ras seems to be required [49-51]. Whether such a combinatorial signaling perturbation will be toxic also for normal cells is a reasonable caveat to be considered.

**Conclusions**

In the last years, substantial progression in the understanding of how mTOR signaling pathway occurs has taught us that there are still significant aspects of mTOR regulation that need further clarification. The mechanism underlying the induction of cell death versus cell-cycle arrest in different cell lines treated with mTOR inhibitors, the existence of additional feedback loops triggered by S6K1 and how these loops affect therapeutic responses should be addressed in the future. Moreover, a clearer picture of the relevance of Akt-dependent versus -independent signaling of PI3K, how do phosphorylation of Akt at Thr308 and at Ser473 influence each other and Akt activity is also needed. Gaining insight in these issues will certainly settle the bases for better therapeutic strategies against cancer.

**References with notes**

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24. Huang J, Dibble CC, Matsuzaaki M, Manning BD. The TSC1-TSC2 complex is required for proper activation of mTOR complex 2. Mol Cell Biol 2008;28:4104–4115. (*) A regulatory role of TSC in mTORC2 activity is shown here. The authors describe that TSC positively regulates mTORC2 activity, hence, also indirectly activating Akt. [PubMed: 18411301]


Figure 1. mTOR signaling pathway

One branch of mTORC1 activation is mediated by the class I phosphoinositide-3 kinase (PI3K), Akt (also known as Pkb) and the tuberous sclerosis complex (TSC). TSC is formed by TSC1 and TSC2, and inhibits a direct activator of mTORC1, the GTPase Ras-homolog enriched in brain (Rheb) by hydrolyzing its GTP into GDP [52]. TSC2 is activated by phosphorylation by AMP-activated protein kinase (AMPK) [53], which is directly activated by a high AMP vs. ATP ratio. AMPK also directly phosphorylates and inactivates Raptor, so it inhibits mTORC1 by TSC-dependent and -independent manners [54]. The activity of AMPK is regulated by phosphorylation by the tumor suppressor LKB1. This protein, like TSC1/2, was found mutated in the germline of patients with different hamartomatous syndromes [55,56]. Akt is a serine/threonine kinase and an important player in regulating mTORC1 activity. Akt positively regulates mTORC1 by acting at different levels. First, Akt inactivates TSC1/2 by phosphorylating TSC2 [57]. Second, Akt inhibits Pras40, negative regulator of mTORC1 that counteracts Rheb function [6,7]. Akt is activated by PI3K, which responds to a variety of growth factors. When activated by insulin or insulin-like growth factors (IGFs), as well as other growth factors, class I PI3K catalyzes the formation of the lipidic second messenger phosphoinositide 3,4,5 tri-phosphate (PIP3) from the bi-phosphate form PIP2. PIP3 triggers
the relocation of Akt to the inner surface of the plasma membrane, where it is activated by phosphoinositide-dependent kinase 1 (PDK1) and transduces the signal as described above. Opposing Akt function is the tumor suppressor Phosphatase and Tensin homolog deleted on chromosome ten (Pten), a lipid phosphatase that converts PIP3 to PIP2, thus shutting off signaling from PI3K. Pten deficiency causes a series of hamartomatous syndromes collectively classified as PTEN hamartoma tumor syndrome (reviewed in [58]). Amino acids activate mTORC1 by an independent route mediated by the Rag family of proteins. The activation of mTORC2 is not well-understood, but this complex directly activates Akt (and Akt-related kinases) by phosphorylation. Akt, in addition, regulates many proteins involved in cell survival and cell cycle progression.
A. S6K inhibition of mTORC1

B. mTORC1, 2, Akt and Deptor

Deptor as a tumor suppressor

Deptor as an oncogene

Curr Opin Cell Biol. Author manuscript; available in PMC 2011 April 1.
Figure 2. Feedback loops modulating mTOR signaling pathway

(A) S6K1 phosphorylation by mTORC1 triggers a number of feedback loops that negatively impact on PI3K signaling. S6K1 directly phosphorylates insulin receptor substrate 1 (IRS-1) at multiple sites, which leads a significant attenuation of insulin and insulin-like growth factor effect at the cell membrane. S6K1 also inhibits PDGFR and the MEK/ERK signaling pathway through a not fully-understood mechanism. These 3 inhibitory loops ultimately dampen PI3K, Akt and mTORC1 activation and have likely evolved as a cellular response to buffer aberrant or excessive PI3K activity. (B) Deptor can have oncogenic and tumor suppressive properties. By blocking mTORC1 and mTORC2, Deptor inhibits protein synthesis, cell size increase and the proliferative and survival effects of Akt. However, under certain conditions, inhibition of mTORC1 by Deptor relieves the feedback inhibition from S6K1 to PI3K, boosting Akt activity. (C) The 2 most important tumor suppressors, namely Pten and p53, cooperate in the inhibition of mTORC1. p53 transactivates many negative regulators of mTORC1 (TSC2, AMPKβ1, Sestrin 1 and 2), which oppose Akt activity. Akt favors degradation of p53 by phosphorylating and stabilizing MDM2. Importantly, Pten and AMPK directly stabilize p53. Collectively, these interactions imply the existence of a coordinate interaction between PI3K, Akt and mTORC1.
regulation of mTOR signaling pathway by energy, nutrients, growth factors, oncogenic stress and DNA integrity.