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Review

Rapamycins

Mechanism of Action and Cellular Resistance

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ABSTRACT

Rapamycins are macrocyclic lactones that possess immunosuppressive, antifungal and antitumor properties. The parent compound, rapamycin, is approved as an immunosuppressive agent for preventing rejection in patients receiving organ transplantation. Two analogues, CCI-779 and RAD001 are currently being investigated as anticancer agents. Rapamycins first bind a cyclophilin FKBP12, and this complex binds and inhibits the function of mTOR (mammalian target of rapamycin) a serine/threonine (Ser/Thr) kinase with homology to phosphatidylinositol 3′ kinase. Currently, as mTOR is the only identified target, this places rapamycins in a unique position of being the most selective kinase inhibitor known. Consequently these agents have been powerful tools in elucidating the role of mTOR in cellular growth, proliferation, survival and tumorigenesis. Increasing evidence suggests that mTOR acts as a central controller sensing cellular environment (nutritional status or mitogenic stimulation) and regulating translation initiation through the eukaryotic initiation factor 4E, and ribosomal p70 S6 kinase pathways. Here we review the conserved TOR signaling pathways, conceptual basis for tumor selectivity, and the mechanisms of resistance to this class of antitumor agent.

INTRODUCTION

Rapamycin, a macrocyclic lactone product of the soil bacteria *Streptomyces hygroscopicus*, was isolated and identified as an antifungal agent in the mid-1970's.[1-3] Rapamycin (sirolimus), is a structural analogue of the macrolide antibiotic FK506 (tacrolimus, Prograf®) (Fig. 1), and like FK506 was found to potently suppress the immune system.[4-7]

The potential for rapamycin to act as an antitumor agent was recognized early in its development when the drug demonstrated potent inhibitory activity against numerous solid tumors in the NCI screening program.[8-10] However, the drug was not developed further due to stability and solubility problems that prevented development of a parenteral formulation for use in clinical trials. Also at that time in the early 1980's, the mechanism of action of rapamycin in blocking signal transduction was not understood.

Rapamycin (Rapamune®), as an immunosuppressive drug, was finally approved by the Food and Drug Administration (FDA) in the USA in 1999, and the European Commission in 2000, respectively. Results from many laboratories have demonstrated that rapamycin, in contrast to FK506, is not only a potent immunosuppressant, but also an active antitumor agent. Rapamycin can act as a cytostatic, slowing or arresting cells in G_1 phase. Under specific conditions, or in some tumor cell lines rapamycins may induce apoptosis in culture. To date, studies have revealed that rapamycin potently arrests growth of cells derived from rhabdomyosarcoma, neuroblastoma and glioblastoma, small cell lung cancer,[11-17] osteoscarcoma,[18] pancreatic cancer,[19,20] breast and prostate cancer,[21-23] murine melanoma and leukemia, and B-cell lymphoma.[9,24-26] In addition to broad spectrum activity in vitro, rapamycin and its derivatives (designated here as rapamycins) suppress growth of some human and murine tumor models in vivo.[11-30] When combined with other chemotherapeutic agents, rapamycins generally show at least additive antitumor activity.[10,12,17,31] Preliminary data from clinical trials have indicated that rapamycins are well tolerated and successfully suppress growth of various human tumors.[32-34]

The use of rapamycin as an anticancer drug is clinically impractical, because of its poor water-solubility and stability in solution. Recently, rapamycin ester analogues (Fig. 1), CCI-779 [rapamycin-42, 2, 2-bis(hydroxymethyl)-propionic acid] (Wyeth-Ayerst, PA, USA) and RAD001 [everolimus, 40-O-(2-hydroxyethyl)-rapamycin] (Novartis, Basel, Switzerland), have entered clinical trials. These analogues have improved pharmaceutical properties. CCI-779 is being developed for both intravenous and oral administration, whereas RAD001 is only for oral administration. The antitumor activity of these analogues

is similar to that of rapamycin.[17,21-23,27-30] RAD001 is in Phase I trials whereas the development of CCI-779 is more advanced with several Phase II trials completed.

Why should it be anticipated that rapamycins could exhibit tumor-selectivity, in a manner analogous to the activity of another kinase inhibitor, Gleevec, in Bcr-Abl expressing chronic lymphocytic leukemia? Accumulating evidence suggests that genetic mutations or compensatory changes in tumor cells may affect sensitivity to rapamycins. At least in some systems mutations that occur frequently in malignant transformations such as *GLI* amplification, or mutations that inactivate p53, and the dual specificity phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome ten, also known as MMAC1 for mutated in multiple advanced cancers) or lead to activation of Akt appear to determine rapamycin sensitivity. On the other hand there may be multiple loci that confer intrinsic or acquired resistance. This review will summarize the current knowledge of the role of mTOR in cellular regulation, the mechanism of action of rapamycins, and currently understood resistance mechanisms.

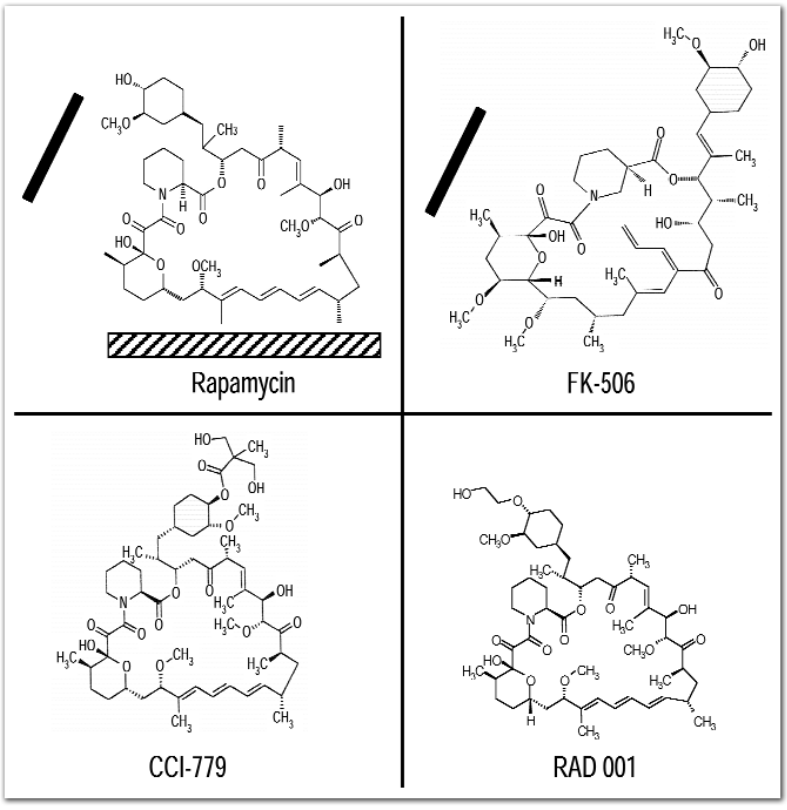

Figure 1. The structure of rapamycin and its analogues FK506, CCI-779 and RAD001. The FKBP12 binding face is shown by the filled bars, whereas the mTOR binding face of rapamycin is shown by the hatched bar.

THE RAPAMYCIN TARGET (MTOR)

The mammalian target of rapamycin, [also named FKBP12 and rapamycin-associated protein (FRAP), rapamycin and FKBP12 target 1 (RAFT1), rapamycin target 1 (RAPT1), or sirolimus effector protein (SEP)], is a 289 kDa Ser/Thr kinase orthologue of TOR1 and TOR2 in *Saccharomyces cerevisiae*.[35,36] TOR is an atypical serine/threonine kinase highly conserved from yeast to mammals. Human, mouse and rat mTOR proteins share 95% identity at the amino acid level.[36-38] Since the C-terminus of TOR is highly homologous to the catalytic domain of phosphatidylinositol 3′ kinase (PI3K), mTOR is considered to belong to the PI3K-related protein kinase (designated PIKK) family, which also includes Mec1, Tel1, RAD3, MEI-41, DNA-PK, ATM, ATR, and TRRAP.[36,37] Recently, single TOR homologs have also been identified in fungi (TOR1 in *Cryptococcus neoformans*), plants (AtTOR in *Arabidopsis thaliana*), worms (CeTOR in *Caenorhabditis elegans*), and flies (dTOR in *Drosophila menalogaster*).[37]

The domain structure of mTOR is depicted in Figure 2. The protein consists of a catalytic kinase domain, an FKBP12-rapamycin binding (FRB) domain and a putative auto-inhibitory domain ("repressor domain") near the C-terminus, and up to 20 tandemly repeated HEAT (Huntingtin, EF3, A subunit of PP2A and TOR) motifs at the N-terminus, as well as FAT (FRAP-ATM-TRRAP) and FATC (FAT C-terminus) domains. HEAT motifs may serve as protein-protein interaction parts, whereas FAT and FATC domains may participate in modulation of catalytic kinase activity of mTOR.[36] The remarkable conservation of mTOR at the amino acid level suggests that multiple domains of this protein are essential for its cellular functions.

Tor Signaling in Yeast. In yeast, Tor kinase activity is regulated by availability of nutrients (amino acids and glucose), whereas in mammalian cells, mTOR is regulated also by phosphatidic acid, ATP, and growth factors.[37,39-42] The Tor signaling pathway in yeast is depicted in Figure 3, and controls translation initiation, protein turnover, transcription, and actin cytoskeleton organization. In yeast these pathways have been rigorously established,[37] and are at least in part maintained in mammalian cells. The Tor1/2 complex (designated TORC1) comprising Kog1 (the yeast homologue of the mammalian protein raptor) and Lst8 controls translation, protein stability and transcription,[43-45] whereas the TORC2 complex controls actin organization. As TORC2 is not a rapamycin target it will not be considered further. The evolutionarily conserved TORC1 complex controls translation initiation probably through activation of eIF4E, and transcription of ribosomal genes, stress response genes, ribosomal biogenesis and tRNA synthesis.

Tor, through the TORC1 complex, controls protein turnover and some aspects of transcription through regulation of protein phos-

Figure 2. Schematic representation of the domains of mTOR. Structural domains of mTOR. HEAT :(huntingtin elongation factor A subunit of PP2A and TOR) repeats (positions 71-522 and 628-1147); FAT: (FRAP-ATM-TRAPP) domain, which is unique to PIK-related kinases located N-terminal to the FKBP12-rapamycin binding domain (FRB); the role of FAT sequences is less clear, but they are associated with C-terminal FAT (FATC) sequences in mTOR. Interaction between FAT and FATC domains may facilitate protein binding or act as a structural scaffold; CD: Catalytic domain; RD: regulatory domain.

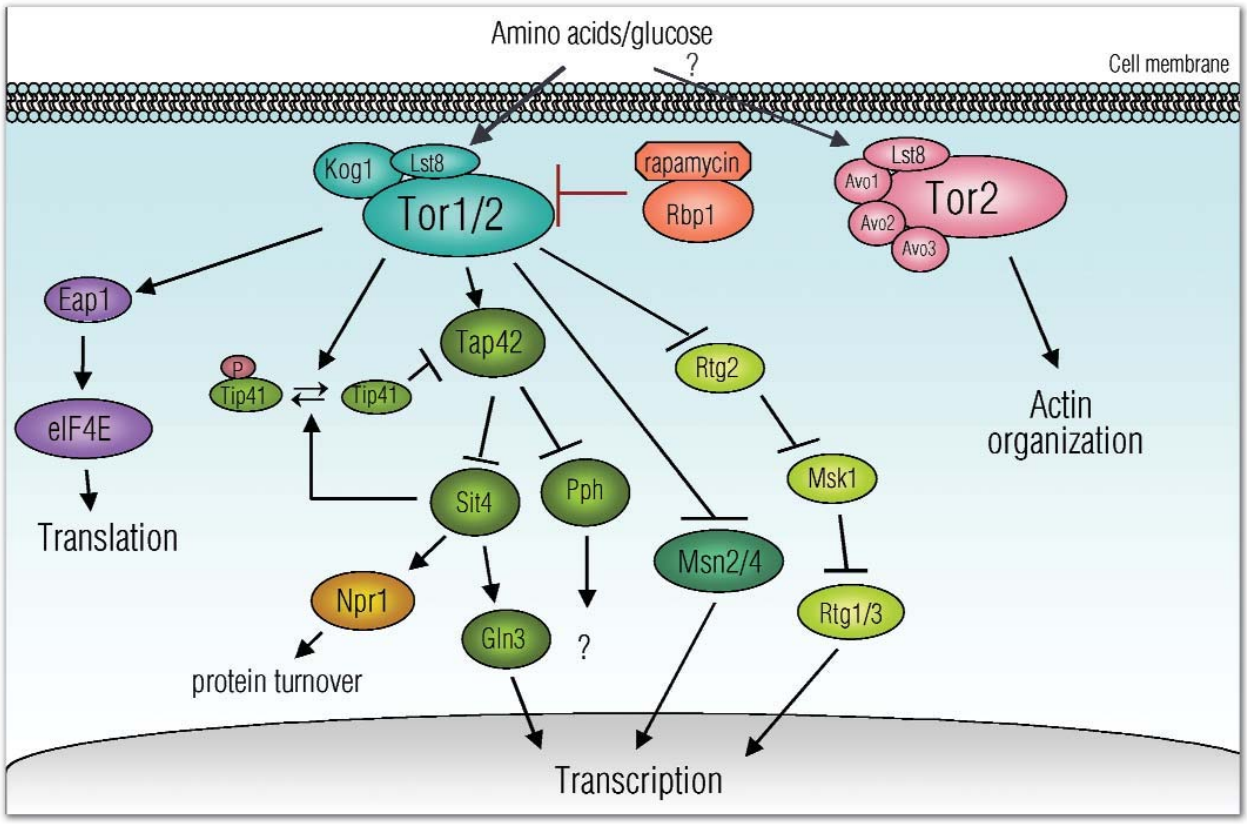

Figure 3. Nutrient signaling in yeast. (Adapted from Jacinto and Hall.[37])

phatases. Tor positively regulates Tap42, which binds to and inactivates the Sit4 and Pph protein phosphatases.[46,47] Pph21 and Pph22 are calaytic subunits of PP2A, and Sit4 is the catalytic subunit of a PP2A-related phosphatase in yeast. Nutrient deficiency or rapamycin leads to dissociation of these phosphatases from Tap42, resulting in increased phosphatase activity. This leads to dephosphorylation of Npr1 and Gln3 involved in protein turnover and transcription, respectively. Control of protein turnover and some aspects of transcription appear to be regulated through Tap42 binding and inactivating Sit4, whereas pathways regulated by release of Pph phosphatases are less well defined. In the presence of nutrients Tor signaling represses autophagy and leads to stabilization of proteins by suppressing ubiquitin-dependent degradation.[48] For example, Tor signaling prevents ubiquitylation, vacuolar targeting and degradation of the tryptophan transporter Tat2 by maintaining phosphorylation and inactivation of Npr1 a putative Tat2 kinase.[49,50] Under conditions of starvation Sit4 becomes activated leading to dephosphorylation of Npr1 and degradation of Tat2. The Tap42-Sit4 pathway also controls the Gln3 transcription factor. Under nutrient replete conditions Gln3 is phosphorylated and is bound to the Ure2 protein in the cytoplasm. Inhibition of Tor by rapamycin or nitrogen starvation leads to dephosphorylation of Gln3, its nuclear translocation and transcription of genes required for the use of secondary nitrogen sources.[43,44] The pathway(s) downstream of Tap42 involving Pph are less clear. Similarly, Tor negatively regulates the heterodimeric transcription factor Rtg1-Rtg3 through an unknown mechanism.[51] The Tor pathway also is important in control of stress-responses through modulation of transcription. The TORC1 complex negatively controls transcription of stress-responsive genes through the cytoplasmic sequestration of the general stress transcription factors Msn2 and Msn4. Although the mechanism is not fully understood this may occur through Tor signaling promoting the binding of these transcription factors to the 14-3-3 homologues Bmh1 and Bmh2.[50]

Proximal Signaling in Mammalian Cells. In mammalian cells, mTOR is regulated not only by nutrients but also by growth factors.[37,39-42] It appears that growth factors regulate mTOR signaling through both PI3K and Akt pathways, whereas proximal activators regulated by nutrients and ATP are less well characterized. In mammalian cells mTOR is activated as a consequence of ligand binding to various growth factor receptors that result in activation of PI3K, Figure 4. Activated PI3K catalyzes the conversion of phostidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). This pathway is negatively regulated by a dual-specificity protein and lipid phosphatase PTEN. Full activation of Akt, downstream of PI3K requires binding of PIP3 to the pleckstrin homology domain of Akt, and phosphorylation by phosphoinositide-dependent kinases 1/2 (PDK1/2) and other unidentified kinases. Pharmacological studies with albeit relatively non-specific inhibitors of PI3K (wortmannin and LY294002) indicate that mTOR is downstream of PI3K. How mTOR is regulated by PI3K or Akt, however, is still not well understood. Akt can phosphorylate mTOR (Ser2448) directly, although the significance remains to be determined.[37,38] Recent studies have placed the tuberous sclerosis (TSC1/2) complex as a modulator between PI3K/Akt and mTOR.[52-54] The TSC1/2 complex comprises harmartin (TSC1) and tuberin (TSC2). These proteins form a physical and functional complex in

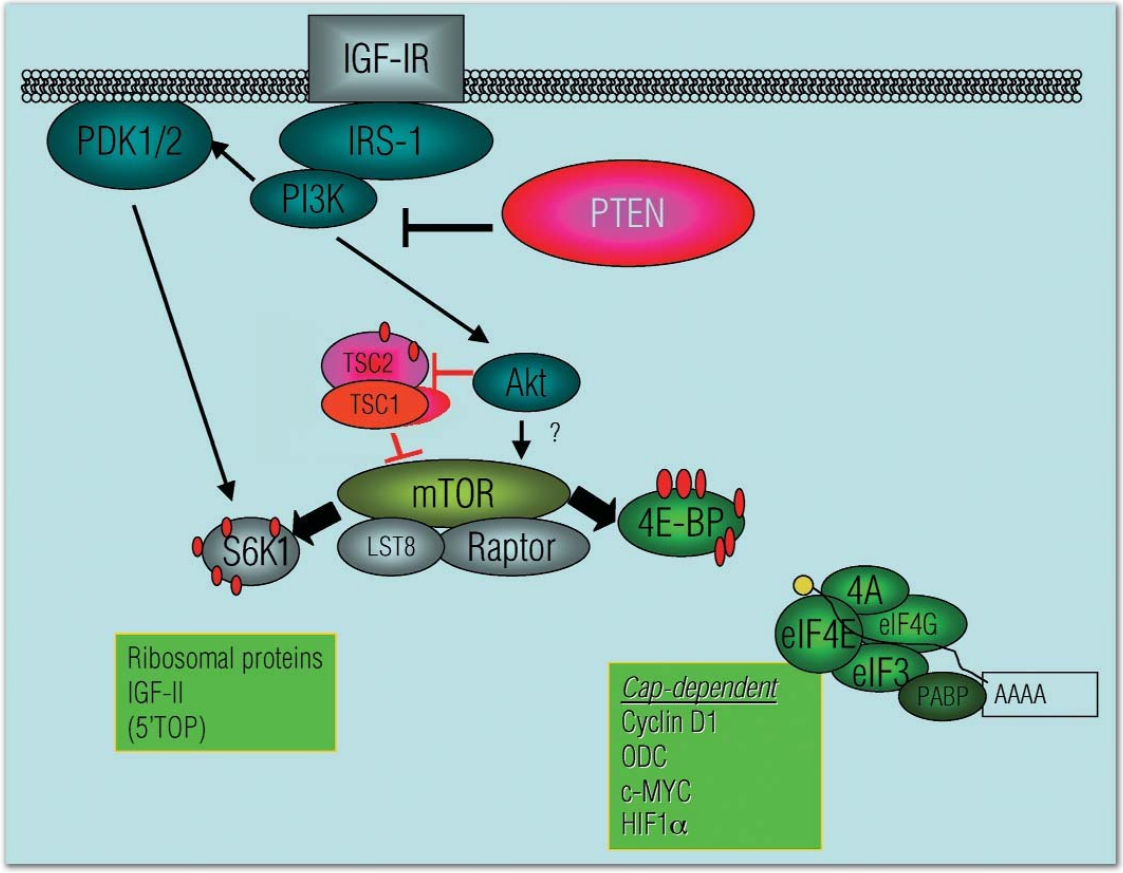

Figure 4. Growth factor signaling in mammalian cells.

vivo, which binds and inhibits mTOR.[52-54] Loss of TSC1/2 results in mTOR-dependent increase of ribosomal p70 S6 kinase (S6K1) activity, and confers resistance of cells to amino acid starvation.[53] Conversely co-expression of TSC1 and TSC2 inhibits amino acid-induced activation of S6K1 in nutrient-deprived cells.[54] Mitogenic stimuli, such as insulin or serum, activate Akt which can directly phosphorylate TSC2 on multiple sites in vitro and in vivo.[52,55,56] Phosphorylation of TSC2 at Ser939 and Thr1462 is PI3K-dependent.[52,56] Akt-mediated phosphorylation of TSC2 destabilizes TSC2 and thereby inhibits the formation of TSC1/2 complex, leading to de-repression of mTOR, and consequently increasing the kinase activity of mTOR. By contrast, treatment with rapamycin does not influence the phosphorylation of TSC2,[52] and together with other data,[53,54] suggests that mTOR lies downstream of TSC2. Other results[57,58] imply that the TSC1/2 complex may mediate S6K1 activation through a pathway parallel to mTOR. However, further studies will be required to address the relationship of mTOR and TSC1/2.

mTOR Associated Proteins. mTOR forms a scaffold complex with other proteins, such as raptor (regulatory associated protein of mTOR) and mLST8,[59,60] which are the mammalian counterparts of yeast Kog1 (kontroller of growth 1) and Lst8,[61] respectively. The exact role of raptor remains unclear.[38,59-61] Raptor may act as a scaffold protein linking mTOR to S6K1 and 4E-BP1.[60] Alternatively, it may have a dual function, inhibiting mTOR under nutrient-deficient conditions and stimulating mTOR in a nutrient-replete environment.[59] Thus TSC1/2, raptor and possibly mLST8 act as potential modulators of mTOR function in response to availability of nutrients.

Signaling Distal to mTOR. mTOR controls translation initiation through two pathways, S6K1 and eukaryotic initiation factor 4E (eIF4E) binding proteins (4E-BPs). mTOR either directly phosphorylates Thr389 of S6K1, or suppresses a phosphatase responsible for maintaining the hypophosphoryalted state of this residue. Activation of S6K1 enhances translation of mRNAs bearing a 5′ terminal oligopyrimidine tract (5′ TOP).[62,63] Inactivation of S6K1 decreases synthesis of ribosomal proteins and translation factors.[62,63] Activation of S6K1 is complex. The process involves interplay between four different domains and at least seven specific sites mediated by multiple upstream kinases.[64] At least 12 sites (Ser17, Thr229, Thr367, Thr371, Thr389, Ser404, Ser411, Ser418, Tr421, Ser424, Ser429, and Thr447) can be phosphorylated in response to serum stimulation.[65] However, the responsible kinases have not been fully characterized. Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates Thr229 in vitro and in vivo.[66] Atypical PKC isoforms and the Rho family of small G proteins (cdc42 and Rac1) may contribute to phosphorylation of S6K1,[65] although the specific sites regulated by these kinases remain to be determined. In vitro, mTOR phosphorylates only Thr389 in the regulatory domain.[67-69] However, whether this phosphorylation is directly or indirectly regulated by mTOR is in question, since recent data suggest that mTOR may regulate S6K1 activation by inhibiting phosphatases rather than directly phosphorylating S6K1.[64,70]

mTOR phosphorylates and inactivates the 4E-BP suppressor proteins causing their dissociation from the RNA cap-binding protein eIF4E. In response to mitogens, six sites (Thr37, Thr46, Ser65, Thr70, Ser83, and Ser112) of 4E-BP1 can be phosphorylated.[65] So far, only mTOR and ATM have been identified to be involved in phosphorylation of 4E-BP1.[71-74] Other kinases that phosphorylate 4E-BP1 remain to be characterized. ATM phosphorylates 4E-BP1 at Ser112,[74] however the physiological significance of this remains to be elucidated. In vitro mTOR phosphorylates 4E-BP1 at two sites (Thr37, Thr46) and possibly at two additional Ser/Thr residues (Thr70 and Ser65) in the N-terminal region.[73,75] Phosphorylation is a hierarchical process.[73,75-77] Phosphorylation of Thr37/Thr46 is followed by Thr70 phosphorylation. Ser65 is phosphorylated last[77] and is dependent on phosphorylation of all three Thr/Pro sites.[75,76] Mutation of Thr37 and/or Thr46 to alanine(s) prevents phosphorylation of Ser65 and Thr70, indicating that phosphorylation of Thr37 and Thr46 serves as a requisite "priming" event.[36] Single phosphorylation of these residues is not sufficient to dissociate 4E-BP1 from eIF4E, indicating the requirement of combined phosphorylation of at least Thr37, Thr46, Ser65, and Thr70 in 4E-BP1 to suppress association with eIF4E.[36,78] Inhibition of mTOR leads to rapid hypophosphorylation of 4E-BP1 which then tightly binds to eIF4E. This prevents formation of eIF4F complex that contains eIF4E, eIF4G, eIF4A and eIF3, and inhibits cap-dependent translation initiation.[36] Once 4E-BP1 is hyperphosphorylated, it releases eIF4E, facilitating eIF4F complex formation and promoting cap-dependent protein synthesis.[36] Overall inhibition of mTOR by rapamycin leads to a decrease in protein synthesis of 15 to 20 percent. However, as the eIF4E pathway is required for translation of mRNA's encoding cyclin D1,[79,80] and ornithine decarboxylase[81] inhibition of mTOR leads to slowing or arrest of cells in G_1 phase of the cell cycle. However, the exact mechanism(s) by which mTOR regulates cell cycle progression are complex, poorly understood, and potentially context specific. Although rapamycins are highly specific inhibitors, the TOR pathway regulates multiple cellular processes. The mTOR pathway regulates translation initiation of survival factors such as c-MYC[82] and hypoxia-inducible factor 1α, and consequently vascular endothelial growth factor.[83,84] In addition, mTOR is involved in the regulation of cyclin A, cyclin dependent kinases (cdk1/2), cdk inhibitors ($p21^{Cip1}$ and $p27^{Kip1}$), retinoblastoma protein, RNA polymerase I/II/III-transcription and translation of rRNA and tRNA, protein phosphatases (PP2A, PP4 and PP6), and CLIP-170.[36,37,85-91]

TUMOR SELECTIVITY OF RAPAMYCINS

To date there are no reports suggesting that activating mutations of mTOR, or overexpression occur as primary events in malignant transformation. However, activation of signaling pathways both proximal and distal to mTOR appear to occur frequently in human cancer. Loss of the phosphatase PTEN by deletion, silencing or mutation leads to constitutive activation of Akt,[29,30,92] and upregulation of mTOR-dependent pathways. In PTEN-deficient tumor cells or mouse embryo fibroblasts (MEFs), activated Akt is associated with enhanced activity of S6K1 and hyperphosphorylation of 4E-BP1,[29,30] or increased levels of c-MYC.[92] It is speculated that Akt-activated cells become dependent on upregulated mTOR signaling for proliferation, hence become more susceptible to rapamycin or CCI-779. Increased sensitivity to rapamycins has been demonstrated in a panel of brain, prostate, and breast cancer cells, multiple myeloma cells and in MEFs.[23,29] The association of PTEN deficiency and sensitivity to rapamycin is further supported by the activity of CCI-779 against the growth of human tumors implanted in athymic nude mice.[23,30] There are, however, some exceptions; cells with functional PTEN and low constitutive activation of Akt are equally sensitive to inhibition of proliferation by rapamycins. Consequently the role of PTEN as an independent variable predicting for rapamycin sensitivity remains to be demonstrated. Disruption of the TSC1 or TSC2 gene leads to the development of tumors in multiple organs, notably kidney brain heart and lung. Even low doses of rapamycin causes apoptosis and necrosis of spontaneous renal tumors in Eker rats with germline mutation in TSC2 and results in tumor regression.[93] These studies suggest that tumors developing in patients with tuberous sclerosis may be sensitive to rapamycins. Oncogene expression may also regulate the response to rapamycin. For example, in RK3E cells transformed with c-MYC or Ras rapamycin treatment increased global protein synthesis. In contrast rapamycin inhibited global protein synthesis and turnover in GLI transformed isogenic lines leading to inhibition of proliferation.[94]

Although generally considered to be cytostatic agents, rapamycins can induce apoptosis in some cell systems. Rapamycins induce apoptosis of B-cells, rhabdomyosarcoma cells, renal tubular cells and dendritic cells.[16,95-97] Rapamycin enhances transforming growth factor-β induced cell cycle arrest,[98] and through blocking survival factor signaling[99] rapamycins enhance cell death. Our results suggest that the functional status of the p53 tumor suppressor may dictate the cellular fate of rapamycin treated cells, as depicted in Figure 5. For example, under serum free conditions, the response to rapamycin in cells lacking functional p53 is apoptosis, suggesting that only in the absence of p53/p21 inhibition of mTOR becomes lethal (so-called synthetic lethality). Ectopic expression of p53 or $p21^{Cip1}$ protects cells from rapamycin-induced apoptosis. The implication is that rapamycins may have potential tumor-selective therapeutic effects.[16] Recent results show that inhibition of mTOR by rapamycin induces a cellular stress response characterized by rapid and sustained activation of ASK1 (apoptosis signal-regulating kinase 1) signaling in p53-mutant cells.[100] This leads to sustained phosphorylation of c-JUN (Ser63) that appears to be responsible for inducing apoptosis. Rapamycin-induced stress appears distinct from that induced by ultra violet radiation in that MEKK1 is not activated, and from cytotoxic agents such as DNA damaging agents where other stress pathways (p38 or ERK1/2) are also activated.[101,102] In contrast, cells expressing wild type p53, (or constitutive $p21^{Cip1}$ expression) there is only transient activation of ASK1.[100] Suppression of ASK1 is associated with binding of $p21^{Cip1}$ in rapamycin treated cells, and protection from apoptosis. Since the rapamycin-induced apoptosis is specifically prevented by insulin-like growth factors (IGF-I/II) and insulin,[96] combination of IGF receptor inhibitors with rapamycins may be selectively cytotoxic and induce regression of tumors with p53 mutations. Whether such anticipation is justified requires vigorous testing.

MECHANISMS OF RESISTANCE TO RAPAMYCINS

Intrinsic sensitivity to rapamycins between cell lines may vary by several orders of magnitude. For example, rhabdomyosarcoma cells in vitro are very sensitive (concentration for 50% inhibition (IC50) of proliferation ~ 1 nM) compared to colon carcinoma cells (IC50 > 5000 nM).[11] Mechanisms of intrinsic and acquired resistance may have either a genetic or epigenetic basis.

Mutations in FKBP12 and mTOR. Rapamycins first bind to the cyclophilin FKBP12 in mammalian cells, forming the FKBP-rapamycin complex. This complex then interacts with the FRB domain in mTOR (Fig. 2), and inhibits the function of mTOR.

Rapamycin resistance may be conferred by mutations in FKBP12 that prevent the formation of FKBP-rapamycin complex, or mutations in the FRB domain of mTOR that block binding of FKBP-rapamycin complex to mTOR. Such mutations were first identified in budding yeast *S. cerevisiae* in which treatment with rapamycin irreversibly arrests cells in the G_1 phase. In the yeast *S. cerevisiae*, deletion of the *RBP1* gene, a homologue of mammalian FKBP-12, results in a recessive rapamycin resistance, whereas expression of *RBP1* restores rapamycin sensitivity.[103] This observation has been further confirmed by RBP1 disruption experiments using the pathogenic yeast *Candida albicans*. Wild-type RBP1/RBP1 parental strain and the rbp1/RBP1 heterozygous mutant were sensitive to rapamycin inhibition, whereas rbp1/rbp1 homozygous mutant was rapamycin resistant.[104] In addition, in *S. cerevisiae* mutation of a specific residue (Tyr89) which is conserved in *RBP1* or FKBPs, also resulted in decreased binding of rapamycin and conferred a recessive resistance phenotype.[105] In murine mast cells, two distinct point mutations in FKBP12 confer resistance. By altering a hydrophobic residue within the drug-binding pocket (Trp59→Leu) or changing a charged surface residue (Arg49→Gln), the binding affinity for rapamycin decreases substantially.[106]

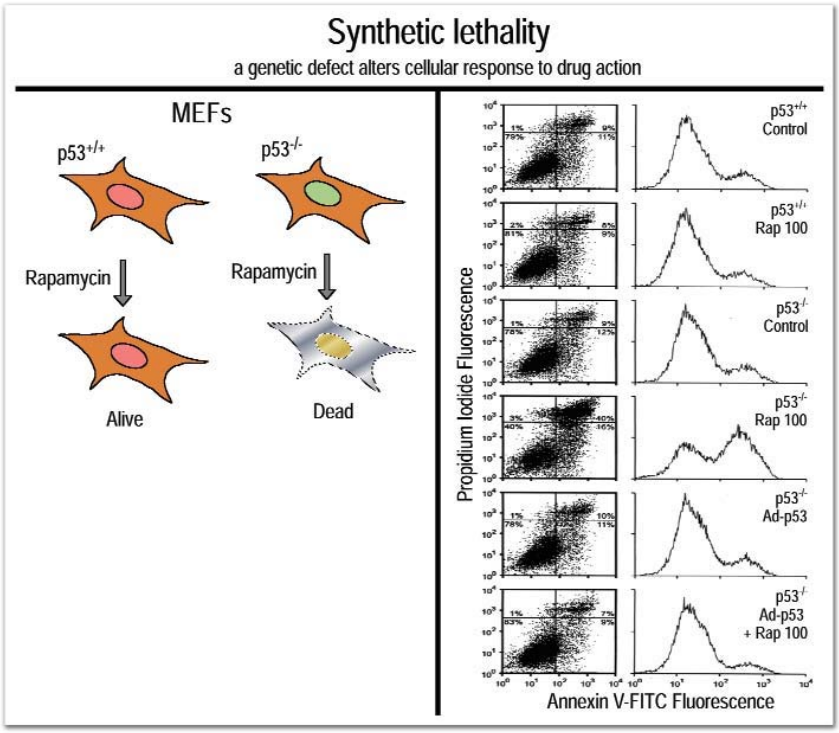

Figure 5. Loss of p53 function alters cellular response to rapamycin from cytostasis to apoptosis in murine embryo fibroblasts (MEFs). Left: schematic representation of synthetic lethality. Right: Wild type, and $p53^{-/-}$ MEFs, and $p53^{-/-}$ MEFs infected withAd-p53 (MOI of 100) were grown without or with rapamycin (100 ng/ml). Cells were harvested after 5 days and apoptosis determined by quantitative FACs analysis (ApoAlert) assay. The per cent distribution of cells in each quadrant is presented (from Huang et al. 2001[16]).

A genetic screen identified rapamycin-resistant alleles with mutations in genes designated *TOR1* and *TOR2*. Strains with mutated to *tor1-1* (Ser1972→Arg) and *tor2-1* (Ser1975→Arg), were completely resistant to the growth- inhibitory effect of rapamycin. These resistant alleles encode mutant Tor proteins that do not bind the FKBP-rapamycin complex.[103,107-111] This result suggests that a conserved serine residue (Ser1972 in Tor1; Ser1975 in Tor2) in Tor proteins is critical for FKBP-rapamycin binding. In mammalian cells mutations in the FRB domain confer a dominant resistant phenotype consistent with decreased affinity for binding of the FKBP- rapamycin complex.[111] Expression of a mutant mTOR (Ser2035→Ile), having greatly reduced binding affinity for the FKBP-rapamycin complex, confers high level resistance.[14,112,113]

Deregulation of eIF4E. mTOR phosphorylates and regulates the function of 4E-BP1, the suppressor of eIF4E.[36] Recently, our group has found that acquired resistance to rapamycin was associated with decreased levels of 4E-BP1.[121] In the absence of selective pressure (rapamycin), resistance was unstable and cells reverted to being sensitive to growth inhibition of rapamycin within ten weeks. In resistant cells the levels of 4E-BP1 were reduced significantly (~10-fold), whereas in rapamycin-sensitive revertants the 4E-BP1 levels increased to those in wild type cells. Levels of 4E-BP1 transcripts were unaltered in rapamycin resistant clones suggesting post-transcriptional regulation. Further studies indicate that the synthesis of 4E-BP1 significantly decreased in rapamycin-resistant clones. Whether the steady state level of 4E-BP1 is also regulated by increased degradation remains to be determined. Thus, the changes in 4E-BP1 levels are reminiscent of those reported for $p27^{Kip1}$ in rapamycin resistant BC3H cells.[115] In contrast, no consistent changes were detected in the level or activity of S6K1 between parental and resistant clones. Rapamycin also inhibited growth factor activation of S6K1 equally in parental and rapamycin-resistant clones. Intrinsic resistance to rapamycin has been shown in glioblastoma cells and colon adenocarcinoma that have very low 4E-BP1.[114] For example, 4E-BP1 is barely detected in HCT8 colon carcinoma cells that are highly resistant to rapamycin (IC50 > 10,000 ng/ml). When 4E-BP1 is overexpressed, these cells become sensitive (IC50 < 10 ng/ml) to rapamycin, Figure 6.[114] These data suggest that low levels of 4E-BP1 results in de-regulation of eIF4E, conferring rapamycin resistance.

These results suggest that rapamycin-regulation of the eIF4E pathway is crucial in inducing growth arrest. Further de-regulation of eIF4E may facilitate a malignant phenotype. Of interest is that both rapamycin-resistant and -revertant cells exhibited elevated c-MYC levels, and increased anchorage-independent growth. That deregulation of the eIF4E pathway is associated with increased malignancy is supported by certain clinical observations that deregulation of the eIF4E pathway does promote tumor progression.[116] In addition to decreased 4E-BP1 expression, as described above, increased eIF4E levels may also cause de-regulation of eIF4E. In advanced head and neck carcinoma,[117] breast carcinoma[118] gastrointestinal carcinoma,[119] and peripheral carcinoma of the lung[120] eIF4E levels are elevated. However, levels of 4E-BP1 suppressor proteins have not been reported in a consistent manner. Potentially, the ratio of 4E-BP:eIF4E may determine whether inhibition of mTOR elicits a biologically significant tumor response. Further studies will be necessary to determine if this ratio has predictive value for drug sensitivity of tumors.

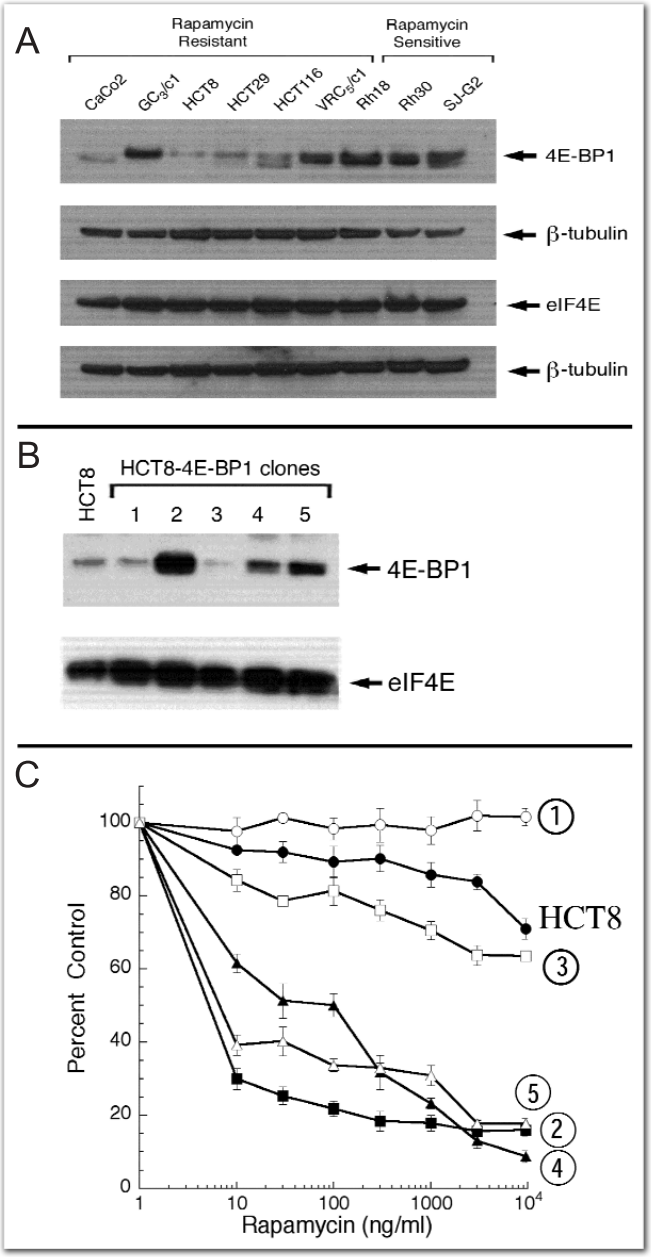

Figure 6. Overexpression of 4E-BP abrogates resistance to rapamycin. (A) Western blot analysis of 4E-BP, eIF4E, and tubulin (loading control) in cell lines that have different intrinsic sensitivities to rapamycin. Colon carcinoma cell lines CaCo2, GC_3/c1, HCT8, HCT29, HCT116, and VRC5/c1 are intrinsically resistant to rapamycin, with IC50 concentrations > 1200 ng/ml. Pediatric solid tumor lines SJ-G2 (glioblastoma) and Rh18 and Rh30 (rhabdomyosarcoma) are sensitive to rapamycin (IC_{50} < 1 ng/ml). (B) Expression of 4E-BP and eIF4E in HCT8 clones stably transfected with a 4E-BP expression plasmid (pcDNA3-PHAS-I). Expression of 4E-BP was greater in clones C2, C4, and C5 than in parental HCT8 cells, but expression of was similar in parental and C1 and C3 transfected clones. (C) Sensitivity to rapamycin. Cells were plated at low density in increasing concentrations of rapamycin, and colonies were counted after 7 days of exposure to rapamycin. Symbols: Parental HCT8 (●) and clones C1 (❍), C2 (■), C3(❑), C4(▲), and C5(Δ). (Adapted from Dilling et al. 2002[114]).

Mutations in S6K1. Ribosomal S6K1 is the other principal downstream effector of mTOR. Inhibition of mTOR by rapamycin primarily inhibits phosphorylation of Thr389 in the regulatory domain.[64] However, since phosphorylation of S6K1 is hierarchical with phosphorylation of several other sites dependent on phosphorylation of Thr389,[64] rapamycin in vivo influences phosphorylation of other sites, including Thr229 in the catalytic domain, and S404 in the regulatory domain.[67] Mutation of Thr389→Glu abrogates the ability of rapamycin to inhibit S6K1 activation.[67,121] Similarly, substitution of Thr229 by either a neutral amino acid Alanine (Thr229→Ala) or by an acidic amino acid Glu (Thr229→Glu), renders S6K1 insensitive to rapamycin.[122] In addition, deletion of the 77 N-terminal codons (ΔN77) confers rapamycin resistance.[123] Of note truncation of the first 54 residues of N-terminus blocks the serum-induced phosphorylation of three rapamycin-sensitive sites, Thr229, Thr389 and Ser404, causing rapamycin insensitivity.[67] Whether this results in resistance to the growth inhibitory effect of rapamycin is less clear, and may be cell context specific.

Mutations of PP2A-Related Phosphatases. The regulation of protein phosphatase activity is thought to be a major mechanism of Tor signaling in yeast. Rapamycin resistance caused by mutations of PP2A-related phosphatases was first studied in *S. cerevisiae*. *PPH21* and *PPH22* encode catalytic-subunits of PP2A (*Pph21* and *Pph22*), whereas *TPD3* and *CDC55* respectively encode the 64 kDa A-subunit and 60 kDa B-subunit. Tap42 is the yeast homologue of mammalian α4, and Sit4 is the yeast homologue of PP6, and the catalytic subunit of a PP2A-related phosphatase in yeast. Under conditions of abundant nitrogen Tor negatively regulates Sit4 by promoting binding to the inhibitor protein Tap42. Under conditions of starvation or rapamycin treatment Sit4 dissociates from Tap42 leading to dephosphorylation of the Gln3 transcription factor and nuclear localization. Strains overexpressing isogenic *tap42-11* mutants were almost completely resistant to rapamycin.[46] In addition, overexpression of Sit4, but not Pph21, also resulted in weak rapamycin resistance.[46] The mechanism of rapamycin resistance in this case is still unknown. More recently a Tap42 interacting protein, Tip41, has been demonstrated to negatively regulate the Tor signaling pathway. Deletion of *TIP41* confers rapamycin resistance, suppresses the *tap42* mutation, and prevents rapamycin-induced dissociation of Sit4 from Tap42.[47] Similarly, mutations or deletion of either *TPD3* (encoding Tpd3, A subunit) or *CDC55* (encoding Cdc55, B subunit), which regulate Pph21/22 activity, conferred rapamycin resistance.[124] These *tpd3* or *cdc55* mutants encode proteins that failed to compete with Tor-phosphorylated Tap42 binding to Pph21/22 catalytic-subunit, resulting in increased association of Tap42 with Pph21/22.[124] These findings indicate that Tap42, Sit4 and PP2A-related phosphatases are downstream effectors of Tor proteins, and mutation or deletion may confer rapamycin resistance.

Several Ser/Thr protein phosphatases, such as PP2A, PP4 and PP6, have been identified as the components of mTOR signaling pathway in mammalian cells.[78] Mammalian PP2A is composed of a common core dimer of a 39 kDa catalytic C-subunit (PP2Ac) and a 65 kDa A-subunit associated with diverse distinct regulatory B-subunits (50-130 kDa). Studies of mammalian cells also indicate that association of α4, the mammalian homologue of Tap42, with PP2A, PP4, and PP6 is related to rapamycin sensitivity.[125,126] For example, in rapamycin-sensitive Jurkat cells, rapamycin caused dissociation of α4 from PP2Ac, whereas in rapamycin-resistant Raji cells, rapamycin did not affect association of α4 with PP2Ac.[126] In Jurkat cells overexpression of α4 conferred rapamycin resistance,[126]

further demonstrating that these PP2A-related phosphatases are novel rapamycin-sensitive targets. Surprisingly, in contrast to results in yeast where dissociation of Sit4 from Tap42 increases phosphatase activity, in mammalian cells rapamycin treatment inhibits cell proliferation by decreasing PP2A activity through dissociating α4 from PP2Ac.[126] This suggests that PP2A may positively regulate cell proliferation under certain conditions. However, other studies[127] do not demonstrate rapamycin-induced dissociation of α4 from PP2A or PP6. Recent data[128] indicate that mTOR may coordinately regulate several phosphatases in rhabdomyosarcoma cells. Rapamycin treatment causes a rapid increase in PP2A activity and a concomitant decrease in PP5 activity. Of interest is that both events are dependent on the expression of 4E-BP1. At this time the significance of α4 remains controversial, however it is clear that in both yeast and mammalian systems alterations in the regulation of protein phosphatases downstream of Tor can confer cellular resistance to rapamycin.

Defective Regulation of $p27^{Kip1}$. In starved cells that are serum stimulated the cyclin dependent kinase inhibitor $p27^{Kip1}$ is downregulated and cells progress through G_1 phase to enter DNA replication. Prevention of mitogen-stimulated downregulation of $p27^{Kip1}$ level by rapamycin suggests that $p27^{Kip1}$ is involved in the antiproliferative activity of rapamycin.[129,130] Rapamycin resistance linked to defective regulation of $p27^{Kip1}$ has been described.[115] Perhaps the most compelling evidence to link $p27^{Kip1}$ and rapamycin sensitivity is the partial resistance to antiproliferative activity of rapamycin found in $p27^{-/-}$ mouse embryo fibroblasts and $p27^{-/-}$ splenic T lymphocytes. Partial resistance may indicate that there are p27-dependent and -independent pathways that determine rapamycin sensitivity. The other association comes from cells with acquired resistance to rapamycin. Prolonged culture of BC3H1 murine myogenic cells in the presence of rapamycin without any induced mutagenesis resulted in rapamycin-resistant clones. Rapamycin resistant cells exhibited abnormally low $p27^{Kip1}$ protein due to a high rate of ubiquitin-independent degradation. Importantly, $p27^{Kip1}$ in these resistant cells could not be regulated, since it was neither reduced in response to serum nor augmented in response to rapamycin. As a result, pRb phosphorylation was blocked by rapamycin in parental MC3H1 cells but not in resistant cells. Rapamycin inhibited proliferation or [^{3}H]-thymidine incorporation to a greater extent in parental BC3H1 cells than in resistant cells. However, in some mammalian cells rapamycin inhibits growth without apparent stabilization of $p27^{Kip1}$. Whether other cdk inhibitors are involved in rapamycin sensitivity remains to be determined.

Mutations of ATM. Recent studies have shown rapamycin resistance in fibroblasts with mutation in the ataxia telangiectasia pathway.[131] ATM (ataxia telangiectasia, mutated) is a 370-kDa protein kinase, which is encoded by the gene mutated in the human genetic disorder ataxia-telangiectasia (A-T) characterized by neuronal degeneration, immunodeficiency, sterility, genomic instability, cancer predisposition, and radiation sensitivity.[132] Like mTOR, the C-terminal sequence of ATM is highly homologous to the catalytic domain of PI3-kinase.[133,134] Three A-T cell lines were significantly more resistant to cell killing by rapamycin than wild type cells, but more sensitive to the PI3-kinase inhibitor wortmannin.[141] Mutations at certain residues of ATM did not determine rapamycin resistance or wortmannin sensitivity, since three A-T cell lines exhibited different mutations in the ATM. Other findings support rapamycin resistance in A-T cells. Rapamycin inhibited cell cycle progression from G1 to S phase in control cells, but failed to prevent cell cycle progression in A-T cells. Consistently, rapamycin decreased phosphorylation of cdk2 and cdk2 kinase activity in wild type cells, but did not affect activation of cdk2 in A-T cells. Although ATM is not the direct target of FKBP-rapamycin complex, a number of other proteins (50–200 kDa) were augmented in their binding to the rapamycin-FKBP complex in the A-T cell lines.[131] The authors proposed that increased resistance to rapamycin in A-T cell lines could be due to alteration in the level of a target protein as a consequence of loss of ATM. In contrast, early passage murine embryo fibroblasts derived from $ATM^{-/-}$ mice are not resistant to rapamycin relative to wild type fibroblasts (our unpublished data). Thus the null phenotype is distinct from the ATM mutant cells. As the ATM cells have genetic instability it is possible that the reported resistance to rapamycin is a consequence of additional mutations and not related directly to defects in ATM signaling.

Mutations of 14-3-3. The 14-3-3 proteins are a highly conserved family of scaffolding and adaptor proteins, with a molecular mass ranging from 27 to 32 kDa. 14-3-3 proteins bind to Ser/Thr-phosphorylated residues in a context specific manner.[135] These proteins participate in cell cycle control, signal transduction and apoptosis by regulating protein-protein interactions, subcellular localization of proteins, and enzyme activity.[136] In *S. cerevisiae*, Bmh1 and Bmh2 are two homologues of the mammalian 14-3-3 proteins and act as multicopy suppressors of the growth-inhibitory phenotype caused by rapamycin.[137] Overexpression of *BMH1* or *BMH2* alone conferred rapamycin resistance, whereas disruption of *BMH1* and/or *BMH2* sensitized the yeast to rapamycin.[137] Interestingly, overexpression of three human 14-3-3 isoforms (β, τ and η) in the yeast also conferred rapamycin resistance. The results suggest that the rapamycin-sensitive function of 14-3-3 proteins is conserved from yeast to human and is isoform-independent. Single or double mutations of *BMH1* (Leu232→Ser and Gly55→Asp) resulted in a dominant rapamycin resistant phenotype. The mechanism by which 14-3-3 proteins cause rapamycin resistance is not known. However, since the yeast Tor proteins lack consensus sites for 14-3-3 binding, Tor proteins could not directly associate with Bmh1p or Bmh2p. Thus, direct interference by Bmh1p and Bmh2p with FKBP-rapamycin binding to Tor1 and Tor2 in the budding yeast may be excluded. Additional studies are necessary to address whether a direct interaction between 14-3-3 proteins and a downstream effector of Tor proteins, or perhaps other mechanisms confer rapamycin resistance.

CONCLUSIONS

The Tor signaling pathway is highly conserved between budding yeast and mammalian cells. In *S. cerevisiae* Tor acts to sense nutritional status, and regulates response to starvation through well defined pathways. In mammalian cells these pathways are less defined. However multi-protein complexes identified in yeast appear conserved in mammalian cells, and some elements of downstream signaling in yeast are recapitulated in higher eukaryotes. Yet, despite extensive studies it remains unclear why inhibition of mTOR results in retardation or arrest of cells in G1 phase of the cell cycle, or in some instances induction of apoptosis. Nor do we yet fully understand why loss of tumor suppressor such as PTEN or p53, or overexpression of certain oncogenes alters the sensitivity of cells to rapamycins. Despite these uncertainties, it is clear that mTOR signaling is critical for proliferation of many cancer cells in vitro, and for tumor growth in vivo. Also of importance is evidence suggesting that mTOR may control certain characteristics of malignancy, such as anchorage-independent growth, and angiogenesis through control of HIF-1α.

However, in this era of molecularly targeted therapies for cancer, it will be important to relate target inhibition to tumor response. As reviewed here, there are several mechanisms of resistance to rapamycin that lie downstream of mTOR. Hence, target inhibition per se may not necessarily relate to cellular response to rapamycins currently under clinical evaluation.

References

1. Vezina C, Kudelski A, Sehgal SN. Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. J Antibiot (Tokyo) 1975; 28:721-26.
2. Sehgal SN, Baker H, Vezina C. Rapamycin (AY-22,989), a new antifungal antibiotic. II. Fermentation, isolation and characterization. J Antibiot (Tokyo) 1975; 28:727-32.
3. Sehgal SN. Rapamune (RAPA, rapamycin, sirolimus): mechanism of action immunosuppressive effect results from blockade of signal transduction and inhibition of cell cycle progression. Clin Biochem 1998; 31:335-40.
4. Calne RY, Collier DS, Lim S, Pollard SG, Samaan A, White DJ, et al. Rapamycin for immunosuppression in organ allografting. Lancet 1989; 2:227.
5. Schreiber SL. Chemistry and biology of the immunophilins and their immunosuppressive ligands. Science 1991; 251:283-87.
6. Pohanka E. New immunosuppressive drugs: an update. Curr Opin Urol 2001; 11: 143-51.
7. Saunders RN, Metcalfe MS, Nicholson ML. Rapamycin in transplantation: a review of the evidence. Kidney Int 2001; 59:3-16.
8. Douros J, Suffness M. New antitumor substances of natural origin. Cancer Treat Rev 1981; 8:63-87.
9. Houchens DP, Ovejera AA, Riblet SM, Slagel DE. Human brain tumor xenografts in nude mice as a chemotherapy model. Eur J Cancer Clin Oncol 1983; 19:799-805.
10. Eng CP, Sehgal SN, Vezina C. Activity of rapamycin (AY-22,989) against transplanted tumors. J Antibiot (Tokyo) 1984; 37:1231-37.
11. Dilling MB, Dias P, Shapiro DN, Germain GS, Johnson RK, Houghton PJ. Rapamycin selectively inhibits the growth of childhood rhabdomyosarcoma cells through inhibition of signaling via the type I insulin-like growth factor receptor. Cancer Res 1994; 54:903-7.
12. Shi Y, Frankel A, Radvanyi LG, Penn LZ, Miller RG, Mills GB. Rapamycin enhances apoptosis and increases sensitivity to cisplatin in vitro. Cancer Res 1995; 55:1982-88.
13. Seufferlein T, Rozengurt E. Rapamycin inhibits constitutive p70 s6k phosphorylation, cell proliferation, and colony formation in small cell lung cancer cells. Cancer Res 1996; 56:3895-97.
14. Hosoi H, Dilling MB, Liu LN, Danks MK, Shikata T, Sekulic A, et al. Studies on the mechanism of resistance to rapamycin in human cancer cells. Mol Pharmacol 1998; 54:815-24.
15. Hosoi H, Dilling MB, Shikata T, Liu LN, Shu L, Ashmun RA, et al. Rapamycin causes poorly reversible inhibition of mTOR and induces p53-independent apoptosis in human rhabdomyosarcoma cells. Cancer Res 1999; 59:886-94.
16. Huang S, Liu LN, Hosoi H, Dilling MB, Shikata T, Houghton PJ. p53/p21^{CIP1} cooperate in enforcing rapamycin-induced G1 arrest and determine the cellular response to rapamycin. Cancer Res 2001; 61:3373-81.
17. Geoerger B, Kerr K, Tang CB, Fung KM, Powell B, Sutton LN, et al. Antitumor activity of the rapamycin analog CCI-779 in human primitive neuroectodermal tumor/medulloblastoma models as single agent and in combination chemotherapy. Cancer Res 2001; 61:1527-32.
18. Ogawa T, Tokuda M, Tomizawa K, Matsui H, Itano T, Konishi R, et al. Osteoblastic differentiation is enhanced by rapamycin in rat osteoblast-like osteosarcoma (ROS 17/2.8) cells. Biochem Biophys Res Commun 1998; 249: 226-30.
19. Grewe M, Gansauge F, Schmid RM, Adler G, Seufferlein T. Regulation of cell growth and cyclin D1 expression by the constitutively active FRAP-S6K1 pathway in human pancreatic cancer cells. Cancer Res 1999; 59:3581-87.
20. Shah SA, Potter MW, Ricciardi R, Perugini RA, Callery MP. Frap-S6K1 signaling is required for pancreatic cancer cell proliferation. J Surg Res 2001; 97: 123-30.
21. Gibbons JJ, Discafani C, Peterson R, Hernandez R, Skotnicki J, Frost P. The effect of CCI-779, a novel macrolide anti-tumor agent, on the growth of human tumor cells in vitro and in nude mouse xenografts in vivo. Proc Am Assoc Cancer Res 1999; 40:301.
22. Yu K, Zhang W, Lucas J, Toral-Barza L, Peterson R, Skotnicki J, et al. Deregulated PI3K/AKT/TOR pathway in PTEN-deficient tumor cells correlates with an increased growth inhibition sensitivity to a TOR kinase inhibitor CCI-779. Proc Am Assoc Cancer Res 2001; 42:802.
23. Yu K, Toral-Barza L, Discafani C, Zhang WG, Skotnicki J, Frost P, et al. mTOR, a novel target in breast cancer: the effect of CCI-779, an mTOR inhibitor, in preclinical models of breast cancer. Endocr Relat Cancer 2001; 8:249-58.
24. Hultsch T, Martin R, Hohman RJ. The effect of the immunophilin ligands rapamycin and FK506 on proliferation of mast cells and other hematopoietic cell lines. Mol Biol Cell 1992; 3:981-87.
25. Gottschalk AR, Boise LH, Thompson CB, Quintans J. Identification of immunosuppressant-induced apoptosis in a murine B-cell line and its prevention by bcl-x but not bcl-2. Proc Natl Acad Sci USA 1994; 91:7350-54.
26. Muthukkumar S, Ramesh TM, Bondada S. Rapamycin, a potent immunosuppressive drug, causes programmed cell death in B lymphoma cells. Transplantation 1995; 60:264-70.
27. Dudkin L, Dilling MB, Cheshire PJ, Harwood FC, Hollingshead M, Arbuck SG, et al. Biochemical correlates of mTOR inhibition by the rapamycin ester CCI-779 and tumor growth inhibition. Clin Cancer Res 2001; 7:1758-64.
28. Beuvink I, O'Reilly T, Zumstein S, Zilbermann F, Sedrani R, Kozma S, et al. Antitumor activity of RAD001, an orally active rapamycin derivative. Proc Am Assoc Cancer Res 2001; 42:366.
29. Neshat MS, Mellinghoff IK, Tran C, Stiles B, Thomas G, Petersen R, et al. Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. Proc Natl Acad Sci USA 2001; 98:10314-19.
30. Podsypanina K, Lee RT, Politis C, Hennessy I, Crane A, Puc J, et al. An inhibitor of mTOR reduces neoplasia and normalizes p70/S6 kinase activity in Pten+/- mice. Proc Natl Acad Sci USA 2001; 98:10320-25.
31. Ishizuka T, Sakata N, Johnson GL, Gelfand EW, Terada N. Rapamycin potentiates dexamethasone-induced apoptosis and inhibits JNK activity in lymphoblastoid cells. Biochem Biophys Res Commun 1997; 230:386-91.
32. Raymond E, Alexandre J, Depenbrock H, Vago AN, Faivre S, Lahr-Randak A, et al. CCI-779, an ester analogue of rapamycin that interacts with PTEN/PI3 kinase pathways: a phase I study utilizing a weekly intravenous schedule. Clin Cancer Res 2000; 6: 4549S.
33. Hidalgo M, Rowinsky E, Erlichman C, Drengler R, Marshall B, Adjei A, et al. Phase I and pharmacological study of CCI-779, a cell cycle inhibitor. Clin Cancer Res 2000; 6:4548S.
34. Hidalgo M, Rowinsky EK. The rapamycin-sensitive signal transduction pathway as a target for cancer therapy. Oncogene 2000; 19:6680-86.
35. Brown EJ, Albers MW, Shin TB, Ichikawa K, Keith CT, Lane WS, et al. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature 1994; 369:756-58.
36. GingrasAC, Raught B, Sonenberg N. Regulation of translation initiation by FRAP/mTOR. Genes Dev 2001; 15:807-26.
37. Jacinto E, Hall MN. Tor signalling in bugs, brain and brawn. Nat Rev Mol Cell Biol 2003; 4:117-26.
38. Abraham RT. Identification of TOR signaling complexes: more TORC for the cell growth engine. Cell 2002; 111:9-12.
39. Fang Y, Vilella-Bach M, Bachmann R, Flanigan A, Chen J. Phosphatidic acid-mediated mitogenic activation of mTOR signaling. Science 2001; 294:1942-45.
40. Dennis PB, Jaeschke A, Saitoh M, Fowler B, Kozma SC, Thomas G. Mammalian TOR: a homeostatic ATP sensor. Science 2001; 294:1102-5.
41. Proud CG. Regulation of mammalian translation factors by nutrients. Eur J Biochem 2002; 269:5338-49.
42. Peng T, Golub TR, Sabatini DM. The immunosuppressant rapamycin mimics a starvation-like signal distinct from amino acid and glucose deprivation. Mol Cell Biol 2002; 22:5575-84.
43. Cardenas ME, Cutler NS, Lorenz MC, Di Como CJ, Heitman J. The TOR signaling cascade regulates gene expression in response to nutrients. Genes Dev. 1999; 13:3271-79.
44. Hardwick JS, Kuruvilla FG, Tong JK, Shamji AF, Schreiber SL. Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins. Proc Natl Acad Sci U S A. 1999 ; 96:14866-70.
45. Powers T, Walter P. Regulation of ribosome biogenesis by the rapamycin-sensitive TOR-signaling pathway in Saccharomyces cerevisiae. Mol Biol Cell. 1999; 10:987-1000.
46. Di Como CJ, Arndt KT. Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases. Genes Dev. 1996; 10:1904-16.
47. Jacinto E, Guo B, Arndt KT, Schmelzle T, Hall MN. TIP41 interacts with TAP42 and negatively regulates the TOR signaling pathway. Mol Cell. 2001; 8:1017-26.
48. Kamada Y, Funakoshi T, Shintani T, Nagano K, Ohsumi M, Ohsumi Y. Tor-mediated induction of autophagy via an Apg1 protein kinase complex. J Cell Biol. 2000; 150:1507-13.
49. Schmidt A, Beck T, Koller A, Kunz J, Hall MN. The TOR nutrient signalling pathway phosphorylates NPR1 and inhibits turnover of the tryptophan permease. EMBO J. 1998; 17:6924-31.
50. Beck T, Schmidt A, Hall MN. Starvation induces vacuolar targeting and degradation of the tryptophan permease in yeast. J Cell Biol. 1999; 146:1227-38.
51. Crespo JL, Powers T, Fowler B, Hall MN. The TOR-controlled transcription activators GLN3, RTG1, and RTG3 are regulated in response to intracellular levels of glutamine. Proc Natl Acad Sci USA. 2002; 99:6784-9.
52. Inoki K, Li Y, Zhu T, Wu J, Guan KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol 2002; 4:648-57.
53. Gao X, Zhang Y, Arrazola P, Hino O, Kobayashi T, Yeung RS, et al. Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. Nat Cell Biol 2002; 4:699-704.
54. Tee AR, Fingar DC, Manning BD, Kwiatkowski DJ, Cantley LC, Blenis J. Tuberous sclerosis complex-1 and 2-gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling. Proc Natl Acad Sci USA 2002; 99:13571-76.
55. Potter CJ, Pedraza LG, Xu T. Akt regulates growth by directly phosphorylating Tsc2. Nat Cell Biol 2002; 4:658-65.
56. Manning BD, Tee AR, Logsdon MN, Blenis J, Cantley LC. Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phoshpoinositide-3-kinase/akt pathway. Mol Cell 2002; 10:151-62.
57. Radimerski T, Montagne J, Hemmings-Mieszczak M, Thomas G. Lethality of *Drosophila* lacking TSC tumor suppressor function rescued by reducing dS6K signaling. Genes Dev 2002; 16:2627-32.

58. Jaeschke A, Hartkamp J, Saitoh M, Roworth W, Nobukuni T, Hodges A, et al. Tumerous sclerosis complex tumor suppressor-mediated S6 kinase inhibition by phosphatidylinositide-3-OH kinase is mTOR independent. J Cell Biol 2002; 159:217-24.
59. Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-Bromage H, et al. MTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell 2002; 110:163-75.
60. Hara K, Maruki Y, Long X, Yoshino K, Oshiro N, Hidayat S, et al. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell 2002; 110:177-89.
61. Loewith R, Jacinto E, Wullschlerger S, Lorberg A, Crespo JL, Bonenfant D, et al. Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol Cell 2002; 10:457-68.
62. Jeffries HB, Fumagalli S, Dennis PB, Reinhard C, Pearson RB, Thomas G. Rapamycin suppressres 5¢TOP mRNA translation through inhibition of S6K1. EMBO J 1997; 16:3693-704.
63. Terada N, Patel HR, Takase K, Kohno K, Nairn AC, Gelfand EW. Rapamycin selectively inhibits translation of mRNAs encoding elongation factors and ribosomal proteins.Proc. Natl. Acad. Sci.1994; 91:11477-81.
64. Volarevic S, Thomas G. Role of S6 phosphorylation and S6 kinase in cell growth. Prog Nucleic Acid Res Mol Biol 2001; 65:101-27.
65. Shah OJ, Anthony JC, Kimball SR, Jefferson LS. 4E-BP1 and : translational integration sites for nutritional and hormonal information in muscle. Am J Physiol Endocrinol Metab 2000; 279:E715-29.
66. Pullen N, Dennis PB, Andjelkovic M, Dufner A, Kozma SC, Hemmings BA, et al. Phosphorylation and activation of S6K1 by PDK1. Science 1998; 279:707-10.
67. Dennis PB, Pullen N, Kozma SC, Thomas G. The principal rapamycin-sensitive S6K1 phosphorylation sites, T-229 and T-389, are differentially regulated by rapamycin-insensitive kinases. Mol Cell Biol 1996; 16:6242-51.
68. Burnett PE, Barrow RK, Cohen NA, Snyder SH, Sabatini DM. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. Proc Natl Acad Sci USA 1998; 95:1432-37.
69. von Manteuffel SR, Dennis PB, Pullen N, Gingras AC, Sonenberg N, Thomas G. The insulin-induced signalling pathway leading to S6 and initiation factor 4E binding protein 1 phosphorylation bifurcates at a rapamycin-sensitive point immediately upstream of S6K1. Mol Cell Biol 1997; 17:5426-36.
70. Peterson RT, Desai BN, Hardwick JS, Schreiber SL. Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FBBP12-rapamycin associated protein. Proc Natl Acad Sci USA 1999; 96:4438-42.
71. Brunn SJ, Hudson CC, Sekulic A, Williams JM, Hosoi H, Houghton PJ, et al. Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. Science 1997; 277:99-101.
72. Hara K, Yonezawa K, Kozlowski MT, Sugimoto T, Andrabi K, Weng QP, et al. Regulation of eIF-4E PB1 phosphorylation by mTOR. J Biol Chem 1997; 272:26567-63.
73. Gingras AC, Gygi SP, Raught B, Polakiewicz RD, Abraham RT, Hoekstra MF, et al. Regulation of 4E-PB1 phosphorylation: a novel two-step mechanism. Genes Dev 1999; 13:1422-37.
74. Yang DQ, Kastan MB. Participation of ATM in insulin signalling through phosphorylation of eIF-4E-binding protein 1. Nat Cell Biol 2000; 2:893-98.
75. Mothe-Satney I, Brunn GJ, McMahon LP, Capaldo CT, Abraham RT, Lawrence JC, Jr. Mammalian target of rapamycin-dependent phosphorylation of PHAS-I in four (S/T)P sites detected by phospho-specific antibodies. J Biol Chem 2000; 275:3386-43.
76. Mothe-Satney I, Yang D, Fadden P, Haystead TA, Lawrence JC Jr. Multiple mechanisms control phosphorylation of PHAS-I in five (S/T)P sites that govern translational repression. Mol Cell Biol 2000; 20:3558-67.
77. Gingras AC, Raught B, Gygi SP, Niedzwiecka A, Miron M, Burley SK, et al. Hierarchical phosphorylation of the translation inhibitor 4E-BP1. Genes Dev 2001; 15:2852-64.
78. Dennis PB, Rumagalli S, Thomas G. Target of rapamycin (TOR): balancing the opposing forces of protein synthesis and degradation. Curr Opin Genet Dev 1999; 9:49-54.
79. Rosenwald IB, Kaspar R, Rousseau D, Gehrke L, Leboulch P, Chen JJ, et al. Eukaryotic translation initiation factor 4E regulates expression of cyclin D1 at transcriptional and post-transcriptional levels. J Biol Chem 1995; 270:21176-80.
80. Hashemolhosseini S, Nagamine Y, Morley SJ, Desrivieres S, Mercep L, Ferrari S. Rapamycin inhibition of the G1 to S transition is mediated by effects on cyclin D1 mRNA and protein stability. J Biol Chem 1998; 273:14424-29.
81. Shantz LM, Pegg AE. Overproduction of ornithine decarboxylase caused by relief of translational repression is associated with neoplastic transformation. Cancer Res 1994; 54:2313-16.
82. DeBenedetti A, Joshi B, Graff JR, Zimmer SG. CHO cells transformed by the translation factor eIF4E display increased c-myc expression but require overexpression of Max for tumorigenicity. Mol Cell Differ. 1994;2:347-71.
83. Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu MM, et al. Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. Cancer Res. 2000;60:1541-5.
84. Mayerhofer M, Valent P, Sperr WR, Griffin JD, Sillaber C. BCR/ABL induces expression of vascular endothelial growth factor and its transcriptional activator, hypoxia inducible factor-1alpha, through a pathway involving phosphoinositide 3-kinase and the mammalian target of rapamycin. Blood. 2002; 100:3767-75.
85. Huang S, Houghton PJ. Inhibitors of mammalian target of rapamycin as novel antitumor agents: from bench to clinic. Curr Opin Investig Drugs 2002; 3:295-04.
86. Braun-Dullaeus RC, Mann MJ, Seay U, Zhantg L, von Der Leyen HE, Morris RE, et al. Cell cycle protein expression in vascular smooth muscle cells in vitro and in vivo is regulated through phosphatidylinositol 3-kinase and mammalian target of rapamycin. Arterioscler Thomb Vasc Biol 2001; 21:1152-58.
87. Thomas G. The S6 kinase signaling pathway in the control of development and growth. Biol Res 2002; 35;305-13.
88. Hudson CC, Liu M, Chiang GG, Otterness DM, Loomis DC, Kaper F, et al. Regulation of hypoxia-inducible factor 1a expression and function by the mammalian target of rapamycin. Mol Cell Biol 2002; 22:7004-14.
89. Treins C, Giorgetti-Peraldi S, Murdaca J, Semenza GL, Van Obberghen E. Insulin stimulates hypoxia-inducible factor 1 through a phosphatidylinositol 3-kinase/target of rapamycin-dependent signaling pathway. J Biol Chem 2022; 277:27975-81.
90. Guba M, von Breitenbuch P, Steinbauer M, Koehl G, Flegel S, Hornung M, et al. Rapamycin-inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. Nat Med 2002; 8:128-35.
91. Choi JH, Bertram PG, Drenan R, Carvalho J, Zhou HH, Zheng XF. The FKBP12-rapamycin-associated protein (FRAP) is a CLIP-170 kinase. EMBO Rep 2002; 3:988-94.
92. Shi Y, Gera J, Hu L, Hsu JH, Bookstein R, Li W, Lichtenstein A. Enhanced sensitivity of multiple myeloma cells containing PTEN mutations to CCI-779. Cancer Res 2002; 62:5027-34.
93. Kenerson HL, Aicher LD, True LD, Yeung RS. Activated mammalian target of rapamycin pathway in the pathogenesis of tuberous sclerosis complex renal tumors. Cancer Res. 2002; 62:5645-50.
94. Louro ID, McKie-Bell P, Gosnell H, Brindley BC, Bucy RP, Ruppert JM. The zinc finger protein GLI induces cellular sensitivity to the mTOR inhibitor rapamycin. Cell growth Differ. 1999; 10:503-16.
95. Lieberthal W, Fuhro R, Andry CC, Rennke H, Abernathy VE, Koh JS, et al. Rapamycin impairs recovery from acute renal failure: a role of cell-cycle arrest and apoptosis of tubular cells. Am J Physiol Renal Physiol 2001; 281:F693-06.
96. Thimmaiah KN, Easton J, Huang S, Veverka KA, Germain GS, Harwood FC, et al. Insulin-like growth factor I-mediated protection from rapamycin-induced apoptosis is independent of Ras-Erk1-Erk2 and phosphatidylinositol 3'-kinase-Akt signaling pathways. Cancer Res 2003; 63:364-74.
97. Woltman AM, Van Der Kooij SW, Coffer PJ, Offringa R, Daha MR, Van kooten C. Rapamycin specifically interferes with GM-CSF signaling in human dendritic cells, leading to apoptosis via increased $p27^{KIP1}$ expression. Blood 2003; 101:1439-45.
98. Law BK, Chytil A, Dumont N, Hamilton EG, Waltner-Law ME, Aarke ME, et al. Rapamycin potentiates transforming growth factor b-induced growth arrest in nontransformed, oncogene-transformed, and human cancer cells. Mol Cell Biol 2002; 22:8184-98.
99. Harada H, Andersen JS, Mann M, Terada N, Korsmeyer SJ. p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD. Proc Natl Acad Sci USA 2001; 98:9666-70.
100. Huang S, Shu L, Dilling MB, Easton J, Harwood FC, Ichijo H, et al. Sustained activation of the JNK cascade and rapamycin-induced apoptosis are suppressed by $p53/p21^{Cip1}$. Mol Cell 2003 (in press).
101. Chang L, Karin M. Mammalian MAP kinase signalling cascades. Nature. 2001; 410:37-40. Review.
102. Osborn MT, Chambers TC. Role of the stress-activated/c-Jun NH2-terminal protein kinase pathway in the cellular response to adriamycin and other chemotherapeutic drugs. J Biol Chem. 1996; 271:30950-5.
103. Heitman J, Movva NR, Hall MN. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. Science 1991; 253:905-09.
104. Cruz MC, Goldstein AL, Blankenship J, Del Poeta M, Perfeect JR, McCusker JH, et al. Rapamycin and less immunosuppressive analogs are toxic to Candida albicans and Cryptococcus neofromans via FKBP12-dependent inhibition of TOR. Antimicrob Agents Chemother 2001; 45:3162-70.
105. Koser PL, Eng WK, Bossard MJ, McLaughlin MM, Cafferkey R, Sathe GM, et al. The tyrosine89 residue of yeast FKBP12 is required for rapamycin binding. Gene 1993; 129:159-65.
106. Fruman DA, Wood MA, Gjertson CK, Katz HR, Burakoff SJ, Bierer BE. FK506 binding protein 12 mediates sensitivity to both FK506 and rapamycin in murine mast cells. Eur J Immunol 1995; 25:563-71.
107. Cafferkey R, Young PR, McLaughlin MM, Bergsma DJ, Koltin Y, Sathe GM, et al. Dominant missense mutations in a novel yeast protein related to mammalian phosphatidylinositol 3-kinase and VPS34 abrogate rapamycin cytotoxicity. Mol Cell Biol 1993; 13:6012-23.
108. Stan R, McLaughlin MM, Cafferkey R, Johnson RK, Rosenberg M, Livi GP. Interaction between FKBP12-rapamycin and TOR involves a conserved serine residue. J Biol Chem 1994; 269:32027-30.
109. Cardenas ME, Heitman J. FKBP12-rapamycin target TOR2 is a vacuolar protein with an associated phosphatidylinositol-4 kinase activity. EMBO J 1995; 14:5892-907.
110. Lorenz MC, Heitman J. TOR mutations confer rapamycin resistance by preventing interaction with FKBP12-rapamycin. J Biol Chem 1995; 270:27531-37.
111. Freeman K, Livi GP. Missense mutations at the FKBP12-rapamycin-binding site of TOR1. Gene 1996; 172:143-47.

112. Dumont FJ, Staruch MJ, Grammer T, Blenis J, Kastner CA, Rupprecht KM. Dominant mutations confer resistance to the immunosuppressant, rapamycin, in variants of a T cell lymphoma. Cell Immunol 1995; 163:70-79.
113. Chen J, Zheng XF, Brown EJ, Schreiber SL. Identification of an 11-kDa FKBP12-rapamycin-binding domain within the 289-kDa FKBP12-rapamycin-associated protein and characterization of a critical serine residue. Proc Natl Acad Sci USA 1995; 92:4947-51.
114. Dilling MB, Germain GS, Dudkin L, Jayaraman AL, Zhang X, Harwood FC, et al. 4E-binding proteins, the suppressors of eukaryotic initiation factor 4E, are down-regulated in cells with acquired or intrinsic resistance to rapamycin. J Biol Chem. 2002; 277:13907-17.
115. Luo Y, Marx SO, Kiyokawa H, Koff A, Massague J, Marks AR. Rapamycin resistance tied to defective regulation of $p27^{Kip1}$. Mol Cell Biol 1996; 16: 6744-51.
116. De Benedetti A, Harris AL. eIF4E expression in tumors: its possible role in progression of malignancies. Int J Biochem Cell Biol 1999; 31:59-72.
117. Nathan CA, Franklin S, Abreo FW, nassar R, De Benedetti A, Glass J. Analysis of surgical margins with the molecular marker eIF4E: a prognostic factor in patients with head and neck cancer. J Clin oncol 1999; 17:2909-14.
118. Li BD, Liu L, Dawson M, De Benedetti A. Overexpression of eurkaryotic initiation factor 4E (eIF4E) in breast carcinoma. Cancer 1997; 79:2385-90.
119. Martin ME, Perez MI, Redondo C, Alvarez MI, Salinas M, Fando JL. 4E binding protein 1 expression is inversely correlated to the progression of gastrointestinal cancers. Int J Biochem Cell Biol 2000; 32:633-42.
120. Seki N, Takasu T, Mandai K, Nakata M, Saeki H, Heike Y, et al. Expression of eukaryotic initiation factor 4E in atypical adenomatous hyperplasia and adenocarcinoma of the human peripheral lung. Clin Cancer Res. 2002; 8:3046-53.
121. Shima H, Pende M, Chen Y, Fumagalli S, Thomas G, Kozma SC. Disruption of the S6K1/p85s6k gene reveals a small mouse phenotype and a new functional S6 kinase. EMBO J 1998; 17:6649-59.
122. Sugiyama H, Papst P, Gelfand EW, Terada N. p70 S6 kinase sensitivity to rapamycin is climated by amino acid substitution of Thr229. J Immunol 1996; 157:656-60.
123. Mahalingam M, Templeton DJ. Constititutive activation of S6 kinase by deletion of amino-terminal autoinhibitory and rapamycin sensitivity domains. Mol Cell Biol 1996; 16:405-13.
124. Jiang Y, Broach JR. Tor proteins and protein phosphatase 2A reciprocally regulate Tap42 in controlling cell growth in yeast. EMBO J 1999; 18:2782-92.
125. Murata K, Wu J, Brautigan DL. B cell receptor-associated protein alpha4 displays rapamycin-sensitive binding directly to the catalytic subunit of protein phosphatase 2A. Proc Natl Acad Sci USA 1997; 94:10624-29.
126. Inui S, Sanjo H, Maeda K, Yamamoto H, Miyamoto E, Sakaguchi N. Ig receptor binding protein 1 (alpha4) is associated with a rapamycin-sensitive signal transduction in lymphocytes through direct binding to the catalytic subunit of protein phosphatase 2A. Blood 1998; 92:539-46.
127. Chen J, Peterson RT, Schreiber SL. a4 associates with protein phosphatases 2A, 4 and 6. Biochim Biophys Res Commun 1998; 247:827-32.
128. Huang S, Shu L, Easton J, Harwood F, Germain G, Houghton PJ. Rapamycin-induced apoptosis: Inhibition of mTOR activates ASK1 signaling by suppression of PP5 activity. Proc. Am. Assoc. Cancer Res.2003; 44: 1598A
129. Nourse J, Firpo E, Flanagan WM, Coats S, Polyak K, Lee MH, et al. Interleukin-2-mediated elimination of the p27Kip1 cyclin-dependent kinase inhibitor prevented by rapamycin. Nature 1994; 372:570-73.
130. Barata JT, Cardoso AA, Nadler LM, Boussiotis VA. Interleukin-7 promotes survival and cell cycle progression of T-cell acute lymphoblastic leukemia cells by down-regulating the cyclin-dependent kinase inhibitor p27Kip1. Blood 2001; 98:1524-31.
131. Beamish H, Williams R, Chen P, Khanna KK, Hobson K, Watters D, et al. Rapamycin resistance in ataxia-telangiectasia. Oncogene 1996; 13:963-70.
132. Shiloh Y, Kastan MB. ATM:genome stability, neuronal development, and cancer cross paths. Adv Cancer Res 2001; 83:209-54.
133. Abraham RT. Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev 2001; 15:2177-96.
134. Savitsky K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L, et al. A single ataxia telangiectasia gene with a product similar to PI-3 kinase. Science 1995; 268:1749-53.
135. Tzivion G, Shen YH, Zhu J. 14-3-3 proteins; bringing new definitions to scaffolding. Oncogene 2001; 20: 6331-38.
136. van Hemert MJ, Steensma HY, van Heusden GP. 14-3-3 proteins: key regulators of cell division, signalling and apoptosis. Bioessays 2001; 23: 936-46.
137. Bertram PG, Zeng C, Thorson J, Shaw AS, Zheng XF. The 14-3-3 proteins positively regulate rapamycin-sensitive signaling. Curr Biol 1998; 8: 1259-67.