Chemistry & Biology
Article

A Chemical Genetic Screen for mTOR Pathway Inhibitors Based on 4E-BP-Dependent Nuclear Accumulation of eIF4E

Mark Livingstone,[1] Ola Larsson,[1] Rami Sukarieh,[1] Jerry Pelletier,[1] and Nahum Sonenberg[1,*]
[1]Department of Biochemistry and McGill Cancer Centre, McGill University, Montreal, QC H3A 1A3, Canada
*Correspondence: nahum.sonenberg@mcgill.ca
DOI 10.1016/j.chembiol.2009.11.010

SUMMARY

The signal transduction pathway wherein mTOR regulates cellular growth and proliferation is an active target for drug discovery. The search for new mTOR inhibitors has recently yielded a handful of promising compounds that hold therapeutic potential. This search has been limited by the lack of a high-throughput assay to monitor the phosphorylation of a direct rapamycin-sensitive mTOR substrate in cells. Here we describe a novel cell-based chemical genetic screen useful for efficiently monitoring mTOR signaling to 4E-BPs in response to stimuli. The screen is based on the nuclear accumulation of eIF4E, which occurs in a 4E-BP-dependent manner specifically upon inhibition of mTOR signaling. Using this assay in a small-scale screen, we have identified several compounds not previously known to inhibit mTOR signaling, demonstrating that this method can be adapted to larger screens.

INTRODUCTION

The mammalian target of rapamycin (mTOR) is a key integrator of multiple intracellular and extracellular cues regulating cell growth and proliferation. Enhanced progrowth mTOR signaling is often seen in cancer resulting from mutations of upstream regulators (e.g., PI3K, PTEN, STK11/LKB1, and TSC2), which uncouple mTOR from environmental cues (Choo and Blenis, 2006; Furic et al., 2009). Consequently, it has been proposed that cancers driven by mTOR signaling would be particularly sensitive to mTOR inhibitory compounds, and as such rapamycin analogs are used in the clinic as anticancer agents (Abraham and Eng, 2008; Choo and Blenis, 2006; Weinstein, 2002).

Rapamycin inhibits mTOR signaling by simultaneously binding to FKBP12 and to the FKBP12-rapamycin binding (FRB) domain of mTOR. mTOR exists in two functionally distinct complexes: the rapamycin-sensitive mTOR complex 1 (mTORC1) and the rapamycin-insensitive mTOR complex 2 (mTORC2) (Loewith et al., 2002; Sarbassov et al., 2004). Recent studies suggested that mTORC1 itself exhibits both rapamycin-sensitive and rapamycin-insensitive activities (Feldman et al., 2009; Thoreen et al., 2009). mTORC1 regulates mRNA translation and cell growth via phosphorylation of the eIF4E binding proteins (4E-BPs) 4E-BP1, 4E-BP2, and 4E-BP3, and of the ribosomal protein S6 (rpS6) kinases, S6K1 and S6K2. The latter belong to the AGC kinase family, together with Akt, various PKC isoforms, and SGK (Mora et al., 2004; Peterson and Schreiber, 1999). Most data support a model whereby mTORC1 phosphorylates 4E-BPs and the hydrophobic motif site on S6K1 and S6K2, whereas mTORC2 is the in vivo kinase for the corresponding site of other key AGC kinase family members (Garcia-Martinez and Alessi, 2008; Hresko and Mueckler, 2005; Ikenoue et al., 2008; Sarbassov et al., 2005). Given the importance of these rapamycin-insensitive mTOR substrates, it has been proposed that non-rapamycin mTOR inhibitors may more potently target mTOR-dependent tumors (Edinger et al., 2003; Sabatini, 2006).

The mechanism by which rapamycin specifically inhibits mTORC1 and not mTORC2 remains elusive. Different theories include (a) an inability of rapamycin to bind mTORC2, possibly due to mTOR's binding partner rictor forming a physical barrier (Jacinto et al., 2004; Sarbassov et al., 2004), (b) the ability of rapamycin to prevent mTOR:raptor complex formation or even to dissociate existing complexes (Hara et al., 2002; Kim et al., 2002; Oshiro et al., 2004), and (c) an mTORC1-specific ability of rapamycin to compete with phosphatidic acid (Toschi et al., 2009). Identification of additional agents that modulate mTORC1 and not mTORC2 activity will facilitate our understanding of this differential mTORC1 versus mTORC2 regulation, and suggest novel inhibitory strategies. The approval of the rapamycin analogs CCI-779 and RAD001 for treatment of patients with renal cell carcinoma is quite promising (Abraham and Eng, 2008). However, because rapamycin treatment can lead to the activation of prosurvival signaling through mTORC2 and Akt (Wan et al., 2007), the tolerance of some cancers to these agents has been postulated to be caused by the inability to target both the mTORC1 and mTORC2 complexes (Rosen and She, 2006). Multiple ATP-competitive mTOR kinase domain inhibitors have been described in the literature that are capable of blocking both mTORC1 and mTORC2 activities (Ballou et al., 2007; Fan et al., 2006; Feldman et al., 2009; Maira et al., 2008; Thoreen et al., 2009). In general, these compounds represent modifications of known PI-3 kinase (PI3K) and PI3K-like kinase (PIKK) inhibitors (Knight et al., 2006), and a limited number of published reports describe more diverse compound library screens (Thoreen et al., 2009; Yang et al., 2007; Yu et al., 2009), due possibly to the cumbersome bacterial expression of the relatively large PIKKs. Therefore, cell-based assays using known in vivo substrates will most likely continue to be the preferred screening method for PIKKs (Fan et al., 2007; Livingstone et al., 2005).

A screen wherein phosphorylation of known mTOR substrates is used as an endpoint is restricted by the availability of suitable antibodies to such phosphoproteins. The phosphoprotein biomarker for mTORC1 signaling, rpS6, also reflects inhibition of S6K1 and non-mTOR inputs to this kinase, whereas antibodies recognizing direct substrates of mTOR (S6K1 and 4E-BP1) have largely proven unsuitable for immunostaining experiments (Engelman et al., 2008; Guertin et al., 2009). Alternative approaches are thus required to monitor mTOR substrate phosphorylation in vivo.

Eukaryotic initiation factor (eIF) 4E, the mRNA 5′ cap-binding protein, is a key regulator of mRNA translation in the cytoplasm, and the activity of eIF4E is repressed by 4E-BPs. Binding of the 4E-BPs to eIF4E is controlled by the mTOR-dependent phosphorylation of 4E-BPs (Gingras et al., 1998; Pause et al., 1994) such that the hypophosphorylated forms of 4E-BPs bind to eIF4E and prevent interaction of eIF4E with eIF4G, thus impairing cap-dependent translation (Haghighat et al., 1995). Conversely, in cells with strong mTOR signaling, 4E-BPs become hyperphosphorylated, releasing eIF4E from 4E-BPs for interaction with eIF4G and assembly into the eIF4F complex. Whereas eIF4E is predominantly cytoplasmic in mammalian cells, nuclear eIF4E has been observed using biochemical fractionation and immunofluorescence analyses (Dostie et al., 2000; Lang et al., 1994; Lejbkowicz et al., 1992), and serum starvation induces an accumulation of eIF4E within the nucleus (Oh et al., 2007). This finding first appeared counterintuitive, because this treatment increases the binding of 4E-BP1 to eIF4E, and biochemical fractionation experiments had previously led to the conclusion that 4E-BP1 localization is restricted to the cytoplasm (Kim and Chen, 2000; Kleijn et al., 2002; Zhang et al., 2002). Using a rabbit monoclonal antibody suitable for immunostaining, we recently showed that 4E-BP1 is present in the nuclei in addition to the cytoplasm of mammalian cells (Rong et al., 2008). Furthermore, we also demonstrated that 4E-BPs are required for the nuclear accumulation of eIF4E seen following serum-starvation and/or rapamycin-treatment: conditions that suppress mTOR signaling (Rong et al., 2008).

The above-described mTOR signaling-modulated and 4E-BP-dependent alteration in eIF4E subcellular localization forms the basis for a novel screening approach. In this report, we demonstrate that cytoplasmic-to-nuclear ratio of eIF4E is a powerful read-out for mTOR signaling and describe a novel cell-based chemical genetic screen that analyzes chemical compound libraries across genetically distinct cell lines. The identification of novel mTOR pathway inhibitory compounds using our small-scale screen may provide new insights into the regulation of mTORC1 versus mTORC2 signaling. It also provides a proof-of-principle that larger screens using this method are possible, both in screening for novel mTOR inhibitory compounds and for counter screens in the search for specific inhibitors of other kinases, particularly PIKKs.

RESULTS

mTOR Pathway Inhibition Is Required for Nuclear Accumulation of eIF4E: Dependence on 4E-BPs

To evaluate the potential of eIF4E localization as a specific marker for mTOR activity we assessed eIF4E localization using immunofluorescence after treatment with known PI3K-mTOR pathway inhibitors (LY294002, wortmannin, rapamycin, and PI-103) or inhibitors of other signaling pathways: U0126, SB203580, and JNK inhibitor II (Bain et al., 2007). Screening for agents that induce a 4E-BP-dependent increase in nuclear eIF4E was performed using both wild-type murine embryonic fibroblasts (MEFs) and 4E-BP1, 4E-BP2 double-knockout (4E-BP1/2 DKO) MEFs, which serve as a negative control. Nuclear accumulation of eIF4E occurred in wild-type, but not in 4E-BP1/2 DKO MEFs, treated with PI3K-mTOR pathway inhibitors (Figure 1 and data not shown). Therefore, inhibition of mTOR signaling resulting in dephosphorylation of nuclear 4E-BPs is necessary for nuclear accumulation of eIF4E. Importantly, the inhibitors of other signaling pathways failed to induce nuclear eIF4E, demonstrating that this readout is specific to mTOR inhibition. Parallel western blot analyses were performed and demonstrated a strong correlation between inhibition of mTOR signaling, dephosphorylation of 4E-BP1, and nuclear accumulation of eIF4E in wild-type but not in 4E-BP1/2 DKO cells (Figure 1). Thus, using immunofluorescence to study eIF4E localization is a powerful assay for in vivo mTOR activity.

Primary Screening to Identify Candidate mTOR Inhibitory Compounds

A screen of 3584 compounds was performed using wild-type MEFs to test the assay for the identification of potentially novel mTOR inhibitors. Chemical libraries used include Prestwick Chemical (1120 structurally diverse marketed drugs), Biomol (361 natural products), Sigma LOPAC (885 pharmacologically active organic compounds), and Microsource Discovery (1218 structurally diverse compounds). For each plate of the 96-well plate-based screen, wells were each treated with one of 80 compounds, while 16 wells were used as negative (dimethyl sulfoxide [DMSO]) and positive (rapamycin) controls. eIF4E immunofluorescent intensity was scored within nuclei as defined by DAPI staining or within a cytoplasmic compartment as defined by a ring outside the nuclei (Figure 2A). For each plate, rapamycin controls were confirmed to induce nuclear accumulation of eIF4E (representative images and values are shown in Figure 2B). Because some apparently toxic compounds dramatically altered cell number, cell morphology, and cell size (Figure 2C and data not shown), these compounds were eliminated prior to data analysis (see Experimental Procedures). Some of these compounds induced significant nuclear accumulation of eIF4E (see Figure S1A available online), and may warrant further characterization. Transformed, normalized and standardized (see Experimental Procedures) cytoplasmic-to-nuclear ratio was used to identify potential mTOR pathway inhibitory compounds. Forty top-scoring compounds were randomly chosen and assessed for their ability to induce dephosphorylation of 4E-BP1 as observed by western blot analysis. Notably, only 20% of the compounds induced a significant decrease in 4E-BP1 phosphorylation, emphasizing the need for secondary screening (Figure S1B).

To directly compare this screening method with immunofluorescence-based monitoring of rpS6 phosphorylation as a read-out of mTOR signaling, IC_{50} values were calculated for the PI3K-mTOR inhibitor LY294002 using both assays and found

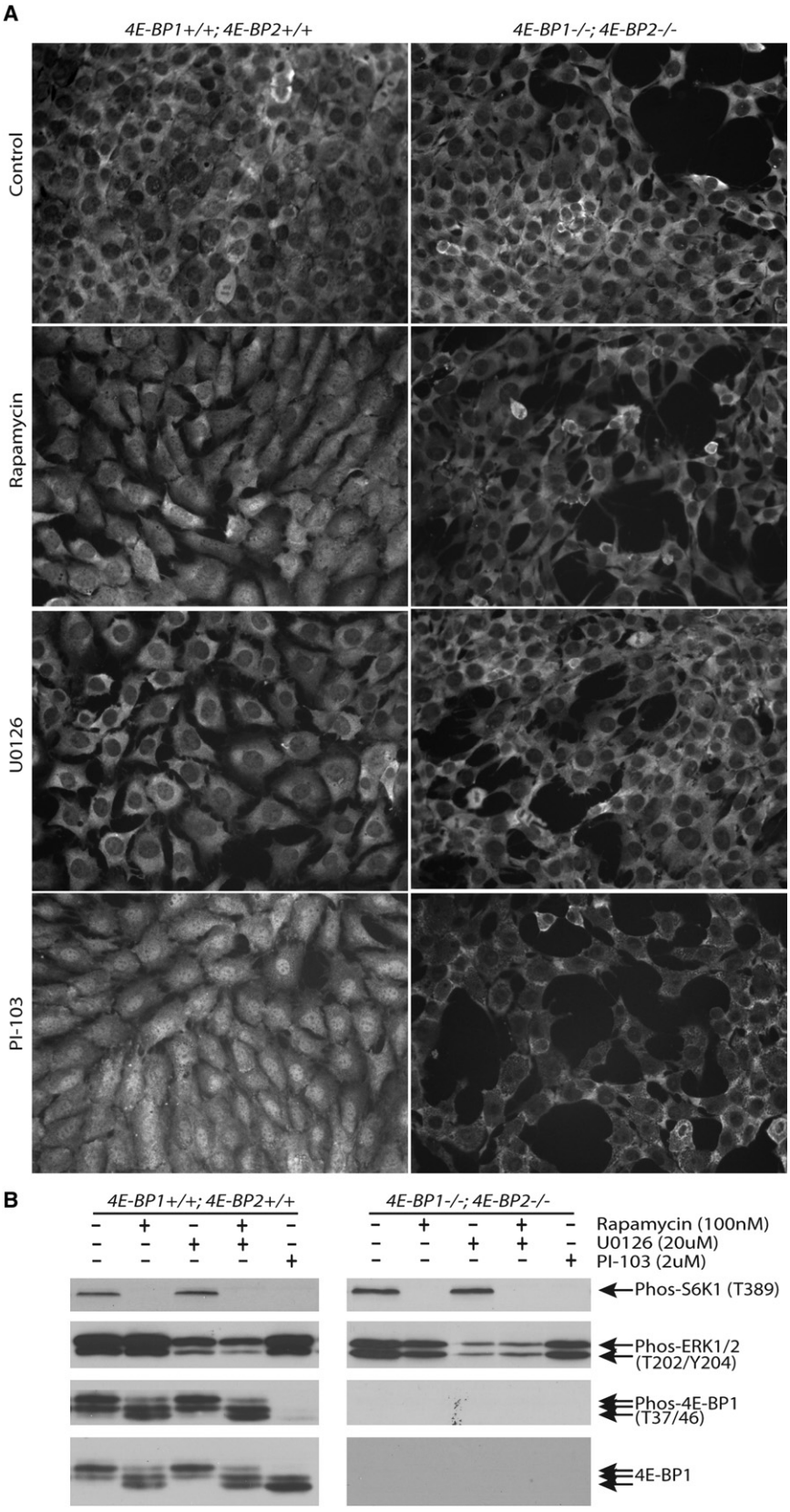


Figure 1. Inhibition of mTOR Signaling Causes 4E-BP-Dependent Nuclear Accumulation of eIF4E

(A) Immunofluorescence analysis of eIF4E localization in response to treatment with rapamycin (100 nM), U0126 (20 μM), or PI-103 (2 μM) reveals that inhibition of the mTOR-PI3K pathway specifically induces nuclear accumulation of eIF4E in wild-type and not 4E-BP1/2 DKO cells.

(B) Western blot analysis demonstrates 4E-BP1 dephosphorylation, as evidenced by accelerated SDS-PAGE mobility and use of phospho-4E-BP1 (Thr37/46) antibody, correlates strongly with nuclear eIF4E. S6K1 (Thr389) and ERK1/2 (Thr202/Tyr204) serve as controls for inhibitor function and specificity.

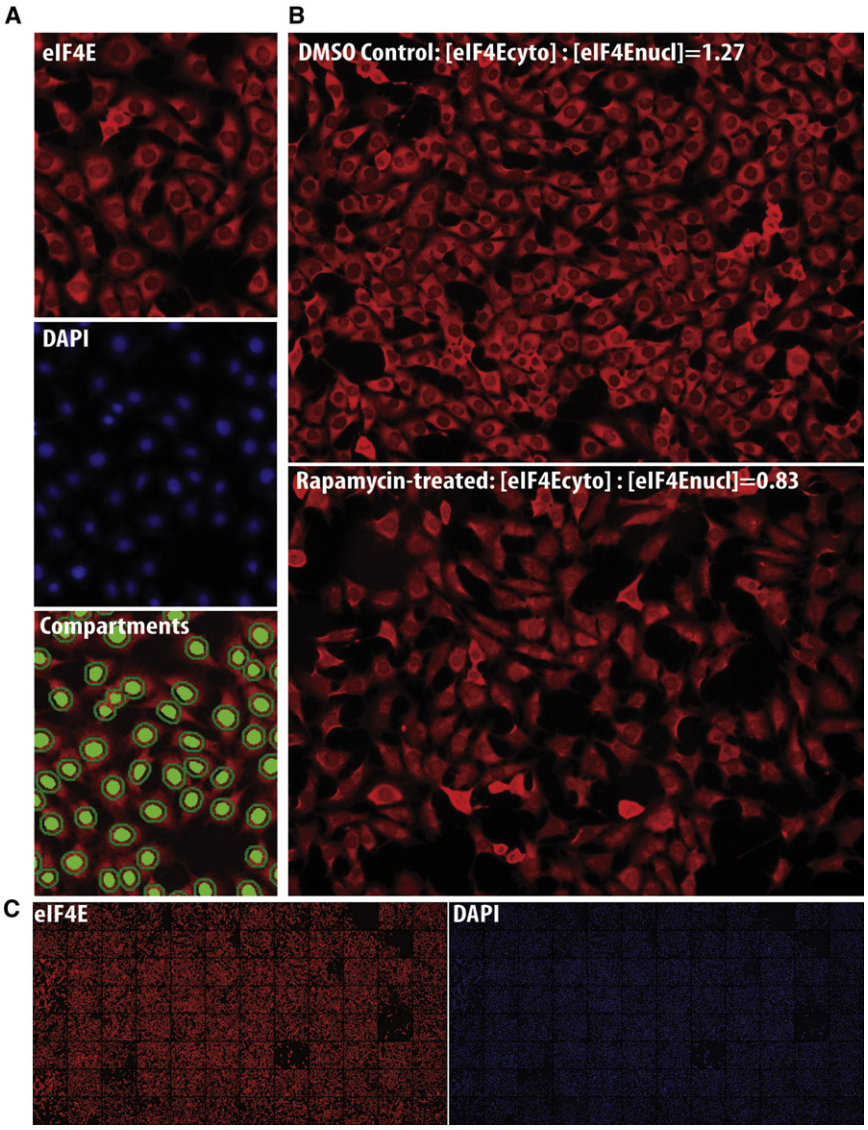


Figure 2. High Content Screen for Novel mTOR Pathway Inhibitors Based on eIF4E Cytoplasmic-to-Nuclear Ratio

(A) Immunofluorescence intensity reflecting eIF4E levels (red) associated with nuclear (light green) or cytoplasmic (dark green) compartments as defined by DAPI staining of DNA (blue).

(B) Representative negative-control (DMSO) and positive-control (rapamycin) images are shown with raw data representing cytoplasmic-to-nuclear eIF4E intensity ratios ([eIF4Ecyto]:[eIF4Enucl]) that reflect the visible accumulation of nuclear eIF4E following rapamycin treatment.

(C) Montage of collected images for a representative 96-well plate demonstrates homogenous staining, but reveals some compounds induce gross morphological and cell number alterations.

to be similar (Figure 3). Maximal inhibition of phospho-rpS6 immunofluorescence intensity and maximal induction of nuclear eIF4E were both observed at a concentration of 50 μM, consistent with previous work demonstrating a greater than 99% inhibition of PI3K at this dose (Vlahos et al., 1994).

Chemical Genetic Screening to Identify mTOR Pathway Inhibitors

The one hundred top-scoring candidate compounds from the primary screen above were subjected to a secondary chemical genetic screen. Because mTOR pathway inhibition-induced nuclear accumulation of eIF4E is dependent on 4E-BPs (Rong et al., 2008), 4E-BP1/2 DKO MEFs were used to eliminate false-positive compounds. As an added control, a homogeneous clonal population of 4E-BP1/2 DKO MEFs rescued by stable expression of HA-4E-BP1 was also included in this secondary screen. Immunofluorescence and western blot characterization of this cell line confirmed that HA-4E-BP1 expression was homogeneous (Figure 4A) and comparable (about one-half) to that of 4E-BP1 in wild-type cells (Figure 4B). Importantly, exogenous HA-tagged 4E-BP1 induced the alteration of eIF4E subcellular localization phenotype in response to mTOR signaling perturbation (Figure 4A).

As expected, the rapamycin control screening wells displayed significantly reduced ($p < 0.001$) eIF4E cytoplasmic-to-nuclear ratio relative to DMSO controls in wild-type but not 4E-BP1/2 DKO cells, and this phenotype was rescued by expression of exogenous HA-4E-BP1 (Figure 4C). Data representing transformed and normalized eIF4E subcellular localization and cytoplasmic compartment size were used as input parameters in a principal components analysis (PCA, see Experimental Procedures). A clear pattern that separated DMSO and rapamycin controls emerged, and only a handful of experimental compounds clustered with rapamycin (Figure 5A). Thus, this method was used to score the compounds, and the top 15 were identified as putative candidates. The individual effects on eIF4E localization across the three cell lines are displayed for the 15 putative candidates and 42 marginally scoring compounds (Figure 5B). As an inadvertent internal positive control, 1 of the 15 identified compounds was rapamycin. The probability of randomly choosing rapamycin as 1 of 15 top-scoring compounds is less than 0.5%. Other identified compounds known to impact signaling pathways include phorbol esters (12-deoxyphorbol 13-phenylacetate 20-acetate and 12-deoxyphorbol 13-acetate) and a non-phorbol ester PKC activator, mezerein. It is not surprising that these, as PKC activators, might reduce 4E-BP1 phosphorylation, because an often-studied phorbol ester/PKC activator, PMA, shows this activity (Guan et al., 2007; Hizli et al., 2006). It should be noted, however, that PMA increases mTOR signaling in some cell lines by inducing p90RSK-dependent inactivation of the mTOR suppressor, TSC2 (Roux et al., 2004).

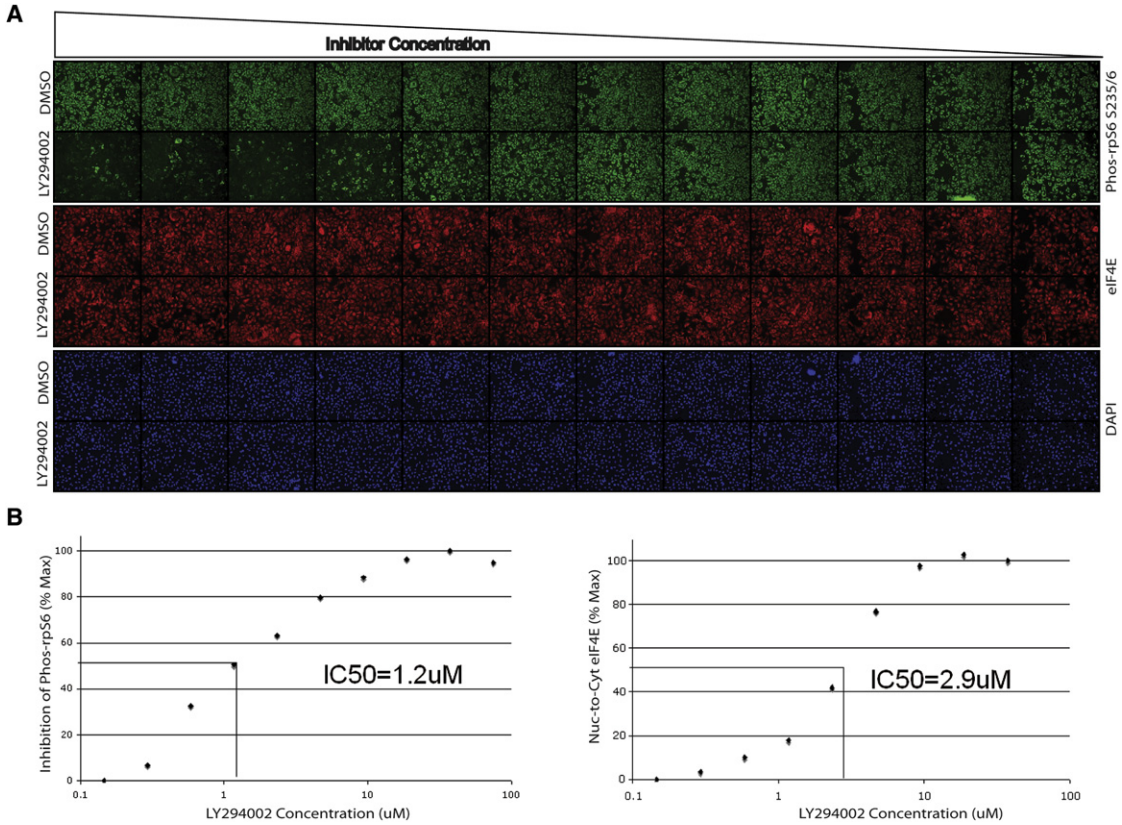


Figure 3. Dose-Response Curves for LY294002 Comparing Phospho-rpS6 (Ser235/236) to Cytoplasmic-to-Nuclear eIF4E Intensity Ratio as Readouts for mTORC1 Signaling

(A) Montage of high content screening images displaying a dose-dependent reduction in phospho-rpS6 immunofluorescence (green) while total eIF4E levels (red) and nuclear staining (blue) remain constant.

(B) LY294002-induced dephosphorylation of rpS6 and eIF4E nuclear accumulation yield comparable dose-response curves and IC_{50} values.

Identified Compounds Inhibit mTORC1 Signaling

A confirmatory immunofluorescence analysis of cells treated with putative candidates using rpS6 phosphorylation as a different marker for mTOR signaling revealed that cells treated with all but one of the compounds (vinblastine sulfate salt) displayed dramatically reduced S6 ribosomal protein phosphorylation, while four moderately scoring compounds showed no significant reduction in S6 phosphorylation (Figure S2A). Western blot analyses confirmed that five compounds (rapamycin, CP107H6, gingerol, fumagillin, and himbacine) dramatically inhibit, five compounds (12-deoxyphorbol 13-phenylacetate, fumonisin B2, e-64-C, ebelactone B, and 12-deoxyphorbol 13-acetate) moderately inhibit, and three compounds (wedelactone, mezerein, and aphidicolin) fail to significantly inhibit mTORC1 signaling to 4E-BP1 and rpS6 in wild-type MEFs and in HeLa S3 cells under the conditions used for the screen (Figure 6A). Further analyses were performed to assess the specificity of seven promising mTORC1 inhibitory compounds using ERK1/2 phosphorylation as a read-out for serum-induced signaling (Figure 6B). Of the tested compounds, ebelactone B, fumagillin, fumonisin B2, and e-64-c failed to block ERK1/2 phosphorylation. To assess the reproducibility of the findings reported here, the four compounds able to inhibit mTOR signaling but not ERK1/2 phosphorylation were purchased, and western blot analysis confirmed that all significantly reduced phosphorylation of 4E-BP1 and S6K1 (Figure S2B).

DISCUSSION

The development of cell-based assays and high-throughput screens for inhibitors of mTOR signaling is intensively pursued. The screen described in this report is particularly powerful and highly adaptable to high-throughput screening because it utilizes a readily monitored change in eIF4E subcellular localization and genetically modified cell lines to measure the in vivo interaction of eIF4E with 4E-BP1 upon suppression of mTOR signaling. Although fluorescence resonance energy transfer (FRET) has been successfully demonstrated in vitro as a means to monitor the interaction of eIF4E with 4E-BP1 (Kimball and Horetsky, 2001), no in vivo counterpart has been described. The use of fluorophore-labeled eIF4E could certainly reduce the manpower required for the eIF4E translocation screen we describe, although the subcellular localization of fluorophore-tagged eIF4E may not recapitulate that of endogenous eIF4E (Kedersha et al., 2005).

This report describes a novel screening method for mTOR pathway activity and by no means represents a comprehensive search for novel mTOR inhibitory compounds. Even within

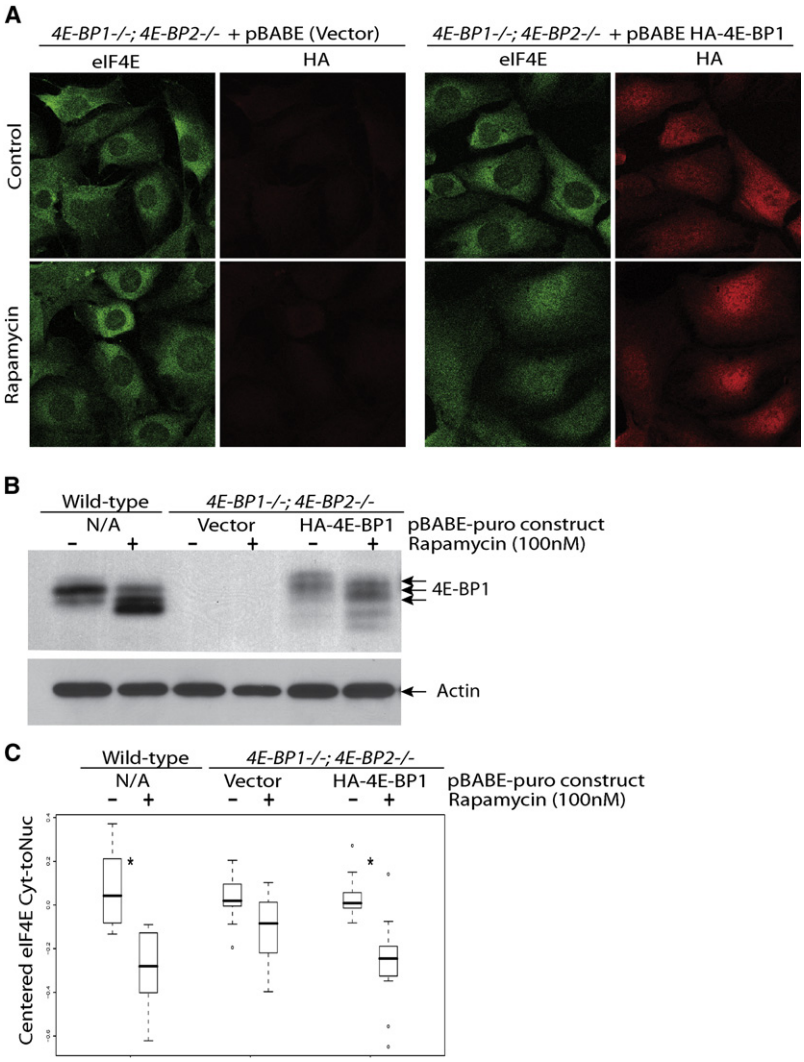


Figure 4. Characterization of the Clonal 4E-BP1 Rescue Cell Line Used for Chemical Genetic Screening

(A) Immunofluorescence analysis of eIF4E and HA-4E-BP1 localization in 4E-BP1/2 DKO cells stably expressing pBABE vector alone or pBABE-HA-4E-BP1 demonstrates rescue of rapamycin-induced nuclear eIF4E accumulation.

(B) Western blot analysis to assess 4E-BP1 levels and rapamycin-induced SDS-PAGE mobility shift. Note: HA-tag retards the apparent SDS-PAGE mobility of 4E-BP1.

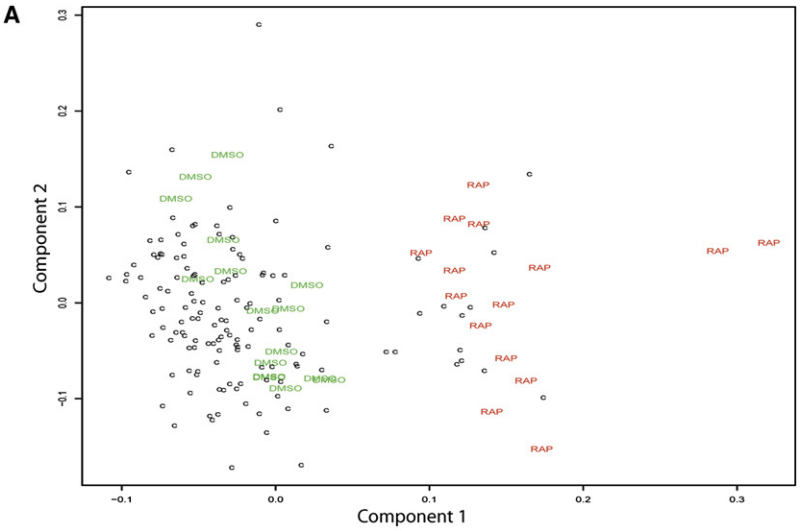
(C) Box plots representing transformed and normalized cytoplasmic-to-nuclear eIF4E intensity values for the negative (DMSO) and positive (rapamycin) control wells ($n = 16$) for each cell line used in the secondary chemical genetic screen. Rapamycin significantly ($p < 0.001$) induced eIF4E nuclear accumulation in wild-type and HA-4E-BP1 rescued cells but not in 4E-BP1/2 DKO cells.

the compound libraries analyzed, mTOR pathway inhibitory compounds will have been missed. A number of compounds were eliminated from candidacy for causing gross morphological or cell number alterations at a concentration of 50 μM. It is possible that some of these could function as excellent, highly potent mTOR pathway inhibitors at lower concentrations. Indeed, the PI3K inhibitors (LY294002 and wortmannin) were both present in the compound libraries screened, but only wortmannin was carried on to the secondary screen. Ultimately, however, both were removed from candidacy due to the aforementioned cell number and cytoplasmic compartment area filters, presumably due to toxicity at the chosen concentration and treatment time. It is noteworthy, however, that rapamycin even at 50 μM (2000 times the standard concentration) was identified as one of the 15 best scoring compounds in this screen. Presumably, this is a reflection of the nontoxicity of this natural product across a broad range of concentrations. Indeed, the bulk of compounds identified as inducing "rapamycin-like" changes in eIF4E subcellular localization in the secondary, chemical genetic screen described above are also natural products.

Although none of the identified compounds was comparable to rapamycin in its ability to specifically inhibit mTOR signaling across a broad range of concentrations, multiple compounds did significantly inhibit mTORC1 signaling under the conditions used for the screen. Although the scope of this study was not aimed at determining the molecular link between each compound and mTOR signaling, known targets of four promising mTORC1 pathway inhibitory compounds identified here merit discussion. Ebelactone B, for example, is a known inhibitor of membrane esterases that may regulate PP2A function (Kowluru et al., 1996; Tan and Rando, 1992). It is also noteworthy that the known PIKK inhibitors wortmannin and LY294002 are also lactones (Konaklieva and Plotkin, 2005), and the lactone oxygen of wortmannin appears to be crucial for competition with ATP (Walker et al., 2000). Fumagillin is known to inhibit methionine aminopeptidase 2 (MetAP2) (Griffith et al., 1997), a potential Ras GTPase activating protein (Xu et al., 1990). Identification of fumagillin as an mTOR pathway inhibitory agent suggests MetAP2 activity may feed into mTOR signaling. Indeed, MetAP2 is upregulated in neurofibromatosis 1 (Nf1)–associated glioma, which is characterized by hyperactive mTOR signaling (Dasgupta et al., 2005a), and proliferation of *Nf1*$^{-/-}$ cells is blocked with fumagillin or rapamycin (Dasgupta et al., 2005b). Fumonisin B2, an inhibitor of sphinganine N-acyltransferase (ceramide synthase) (Wang et al., 1991), was also identified and may suggest a link between sphingolipid metabolism and mTOR signaling. Finally, the calpain inhibitor E-64-c also reduced mTOR signaling, which is unexpected in light of previous evidence that calpain itself is inhibitory to Akt and mTOR signaling (Smith and Dodd, 2007).

SIGNIFICANCE

The mammalian target of rapamycin (mTOR) has proven to be a therapeutically beneficial drug target due to its



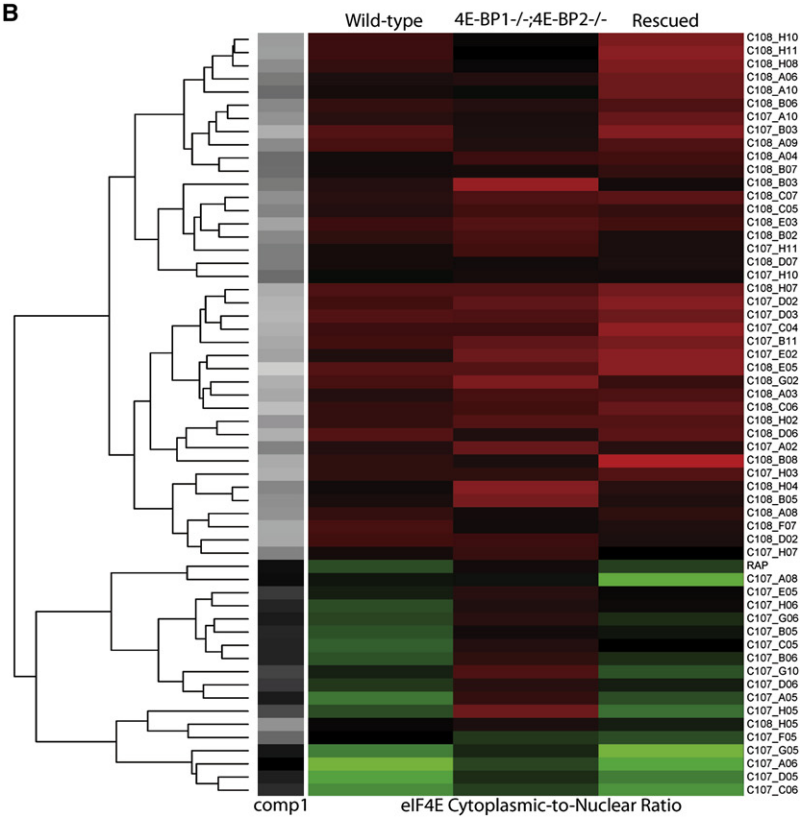


Figure 5. Putative Candidate mTORC1 Pathway Inhibitors Identified by Chemical Genetic Screening

(A) Principal components analysis of eIF4E localization and cytoplasmic compartment size in response to candidate compounds across the three cell lines. Compounds (c) cluster either with rapamycin (RAP) or with DMSO controls primarily based on component 1 (*x* axis).

(B) Heat map of compounds with a transformed and normalized cytoplasmic-to-nuclear eIF4E intensity ratio < 0 in WT cells. Shown is score from PCA component 1 (as a gray scale with high score indicated by black) for compounds clustered based on eIF4E cytoplasmic-to-nuclear localization as indicated and cytoplasmic compartment area (data not shown) across the three cell lines. Green indicates a reduced cytoplasmic-to-nuclear eIF4E intensity ratio, while red indicates opposite effect, with black representing an intermediate effect.

phylogenetically conserved role of integrating intracellular and extracellular signals to modulate cellular growth and proliferation. In this report we provide strong proof-of-principle data illustrating the power and usefulness of a novel cell-based assay for compounds that impact mTOR signaling. The readout for this screen is the nuclear accumulation of the mRNA 5′ cap binding protein eIF4E, which we demonstrate to occur specifically upon inhibition of mTOR-dependent phosphorylation of 4E-BPs. This chemical genetic screen makes use of genetically modified cell lines, thus reducing the possibility of false positives, including autofluorescent compounds. Given the ongoing efforts toward development of mTOR and PIKK inhibitors, this screen can directly be used with much larger chemical libraries to identify novel classes of mTOR pathway inhibitory molecules. The compounds identified in our small-scale screen as specifically inhibiting mTORC1 signaling to 4E-BP1 and S6K1 provide new information regarding the regulation of mTOR signaling and may form the basis of novel strategies to target this important regulator of cell growth and proliferation.

EXPERIMENTAL PROCEDURES

The wild-type and *4E-BP1*$^{-/-}$*; 4E-BP2*$^{-/-}$ MEFs have been previously described, as has the pBABE-puro-HA-4E-BP1 rescue cell line (Rong et al., 2008). The subcellular localization of eIF4E was monitored by indirect immunofluorescence (anti-mouse IgG Alexa647, 1:200, Invitrogen) colocalization with DAPI (200 ng/ml)-stained nuclei, wherein cells were washed with cold phosphate-buffered saline (PBS), prior to fixation with formaldehyde (3.7% in PBS, 37°C, 10 min) and permeabilization with methanol (100%, −20°C) followed by blocking in bovine serum albumin (BSA) (2% in PBS) and overnight incubation with eIF4E mAb (1:400 in 2% BSA, BD Transduction Labs). Commercially available compound libraries (Prestwick, Biomol, MicroSource Discovery, and Sigma LOPAC) were used for screening at 50 μM for 5 hr. This and similar drug concentrations have previously been shown to be optimal for inducing nuclear accumulation of the transcription factor FOXO1A using high content screening (Haney, 2008; Kau et al., 2003), and hence was chosen as the starting concentration for subsequent screening experiments. Additional compounds including PI-103, JNK inhibitor II (EMD Biosciences), LY294002, Wortmannin, U0126 (Sigma-Aldrich), Rapamycin, Swainsonine, Fumagillin, E-64-c, Fumonisin B2, and Ebelactone B (Biomol) were purchased separately for controls and validation screening.

For all screening experiments, cytoplasmic-to-nuclear eIF4E intensity ratios (MetaXpress® Translocation-Enhanced) for each well of 96-well plates were collected, as were compartment size and cell number values. All data analysis was performed in R (www.r-project.org). For both the primary and the

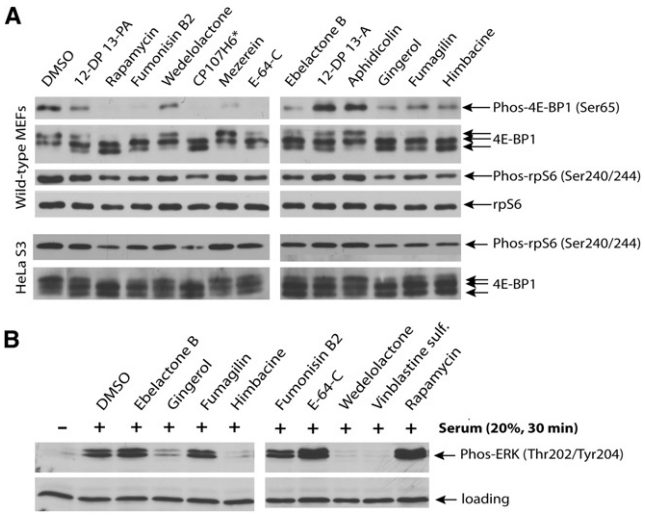


Figure 6. Western Blot-Based Characterization of Top-Scoring Compounds

(A) The majority of top-scoring compounds (50 μM, 5 hr) significantly reduce 4E-BP1 and rpS6 phosphorylation in wild-type MEFs (upper) and in HeLa S3 cells (lower). The phorbol esters 12-deoxyphorbol 13-phenylacetate (12-DP 13-PA) and 12-deoxyphorbol 13-acetate (12-DP 13-A) induce intermediate effects.

(B) Western blot assessment of the impact of these compounds (2 μM, 1 hr) on serum-stimulated (20%, 30 min) ERK phosphorylation.

secondary screen, data associated with compounds causing extreme changes in compartment size (<75% mean) or cell number (<25% mean) were discarded and did not participate in data transformation and normalization. For the first screen, cytoplasmic-to-nuclear eIF4E intensity ratios were log2 transformed and subjected to normalization plate by plate after removing positive and negative controls using the trimmed polish approach, which is a modification of the median polish approach using the mean instead of the median, after removing top and bottom 10% (per row or column). This approach identified and removed both row and column bias. The data were further standardized using the median absolute deviation per plate. This transformed, normalized, and standardized data set was used to create a ranking of all compounds, and the immunofluorescence images associated with top 150 scoring compounds (approximately corresponding to standardized values < -2) were subjected to visual inspection to eliminate compounds generating gross morphological alterations. For the secondary screen, a separate data analysis approach was used, because the approach described for the first screen relies on the presence of a limited set of hits per plate, whereas the second round screening could contain more hits that may be artificially removed by the trimmed polish approach. The compartment size and the cytoplasmic-to-nuclear eIF4E intensity ratios were log2 transformed and centered (mean 0) per plate. The resulting data set (three cell types, both cytoplasmic-to-nuclear eIF4E intensity ratios, and cytoplasmic compartment area) was used in a PCA. Compounds showing a transformed normalized cytoplasmic-to-nuclear eIF4E intensity ratio < 0 in the wild-type cell line were subjected to hierarchical clustering while indicating the score from the PCA analysis.

Western blot (phospho-S6K1 T389, phospho-ERK1/2 T202/Y204, phospho-rpS6 S240/244, 4E-BP1, phospho-4E-BP1 T37/46 ,and phospho-4E-BP1 S65) and immunostaining analyses (phospho-rpS6 S235/6) were performed according to the manufacturer's (Cell Signaling Technology) recommended protocols. 4E-BP1 phosphorylation was assessed using phospho-specific antibodies or by calculating relative band intensities (ImageJ) for hyperphosphorylated (upper band) and hypophosphorylated (lower band). Alexa488 and Alexa647 conjugated secondary antibodies (Invitrogen) were used for indirect immunofluorescence. Confocal images were obtained using a 63X objective of a Zeiss LSM 510 confocal microscope. Additional antibodies used for immunostaining experiments were HA-tag (16B12) mAb (Covance), and eIF4E rabbit mAb (Cell Signaling Technology).

SUPPLEMENTAL DATA

Supplemental Data include two figures and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00401-3.

ACKNOWLEDGMENTS

This research was supported by a National Cancer Institute of Canada Grant (to N.S.) and by a CIHR Team Grant (#CTP-79858) on the Molecular Basis of Translational Control of Memory Formation (to J.P.). We thank Xiaofeng Wang for technical assistance with screening experiments and the McGill Life Sciences Complex Imaging Facility for assistance with confocal microscopy. M.L. and R.S. were supported by McGill-CIHR Chemical Biology Studentships. M.L. is a Research Student of the Terry Fox Foundation (Award #700029).

Received: August 6, 2009
Revised: October 20, 2009
Accepted: November 13, 2009
Published: December 23, 2009

REFERENCES

Abraham, R.T., and Eng, C.H. (2008). Mammalian target of rapamycin as a therapeutic target in oncology. Expert Opin. Ther. Targets *12*, 209–222.

Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C.J., McLauchlan, H., Klevernic, I., Arthur, J.S., Alessi, D.R., and Cohen, P. (2007). The selectivity of protein kinase inhibitors: a further update. Biochem. J. *408*, 297–315.

Ballou, L.M., Selinger, E.S., Choi, J.Y., Drueckhammer, D.G., and Lin, R.Z. (2007). Inhibition of mammalian target of rapamycin signaling by 2-(morpholin-1-yl)pyrimido[2,1-alpha]isoquinolin-4-one. J. Biol. Chem. *282*, 24463–24470, Epub *22007*, 24411.

Choo, A.Y., and Blenis, J. (2006). TORgeting oncogene addiction for cancer therapy. Cancer Cell *9*, 77–79.

Dasgupta, B., Yi, Y., Chen, D.Y., Weber, J.D., and Gutmann, D.H. (2005a). Proteomic analysis reveals hyperactivation of the mammalian target of rapamycin pathway in neurofibromatosis 1-associated human and mouse brain tumors. Cancer Res. *65*, 2755–2760.

Dasgupta, B., Yi, Y., Hegedus, B., Weber, J.D., and Gutmann, D.H. (2005b). Cerebrospinal fluid proteomic analysis reveals dysregulation of methionine aminopeptidase-2 expression in human and mouse neurofibromatosis 1-associated glioma. Cancer Res. *65*, 9843–9850.

Dostie, J., Ferraiuolo, M., Pause, A., Adam, S.A., and Sonenberg, N. (2000). A novel shuttling protein, 4E-T, mediates the nuclear import of the mRNA 5′ cap-binding protein, eIF4E. EMBO J. *19*, 3142–3156.

Edinger, A.L., Linardic, C.M., Chiang, G.G., Thompson, C.B., and Abraham, R.T. (2003). Differential effects of rapamycin on mammalian target of rapamycin signaling functions in mammalian cells. Cancer Res. *63*, 8451–8460.

Engelman, J.A., Chen, L., Tan, X., Crosby, K., Guimaraes, A.R., Upadhyay, R., Maira, M., McNamara, K., Perera, S.A., Song, Y., et al. (2008). Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. Nat. Med. *14*, 1351–1356.

Fan, Q.W., Knight, Z.A., Goldenberg, D.D., Yu, W., Mostov, K.E., Stokoe, D., Shokat, K.M., and Weiss, W.A. (2006). A dual PI3 kinase/mTOR inhibitor reveals emergent efficacy in glioma. Cancer Cell *9*, 341–349.

Fan, Q.W., Cheng, C.K., Nicolaides, T.P., Hackett, C.S., Knight, Z.A., Shokat, K.M., and Weiss, W.A. (2007). A dual phosphoinositide-3-kinase alpha/mTOR inhibitor cooperates with blockade of epidermal growth factor receptor in PTEN-mutant glioma. Cancer Res. *67*, 7960–7965.

Feldman, M.E., Apsel, B., Uotila, A., Loewith, R., Knight, Z.A., Ruggero, D., and Shokat, K.M. (2009). Active-Site Inhibitors of mTOR Target Rapamycin-Resistant Outputs of mTORC1 and mTORC2. PLoS Biol. *7*, e38.

Furic, L., Livingstone, M., Dowling, R.J., and Sonenberg, N. (2009). Targeting mtor-dependent tumours with specific inhibitors: a model for personalized medicine based on molecular diagnoses. Curr. Oncol. *16*, 59–61.

Garcia-Martinez, J.M., and Alessi, D.R. (2008). mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). Biochem. J. *416*, 375–385.

Gingras, A.C., Kennedy, S.G., O'Leary, M.A., Sonenberg, N., and Hay, N. (1998). 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. Genes Dev. *12*, 502–513.

Griffith, E.C., Su, Z., Turk, B.E., Chen, S., Chang, Y.H., Wu, Z., Biemann, K., and Liu, J.O. (1997). Methionine aminopeptidase (type 2) is the common target for angiogenesis inhibitors AGM-1470 and ovalicin. Chem. Biol. *4*, 461–471.

Guan, L., Song, K., Pysz, M.A., Curry, K.J., Hizli, A.A., Danielpour, D., Black, A.R., and Black, J.D. (2007). Protein kinase C-mediated down-regulation of cyclin D1 involves activation of the translational repressor 4E-BP1 via a phosphoinositide 3-kinase/Akt-independent, protein phosphatase 2A-dependent mechanism in intestinal epithelial cells. J. Biol. Chem. *282*, 14213–14225, Epub *12007*, 14213.

Guertin, D.A., Stevens, D.M., Saitoh, M., Kinkel, S., Crosby, K., Sheen, J.H., Mullholland, D.J., Magnuson, M.A., Wu, H., and Sabatini, D.M. (2009). mTOR complex 2 is required for the development of prostate cancer induced by Pten loss in mice. Cancer Cell *15*, 148–159.

Haghighat, A., Mader, S., Pause, A., and Sonenberg, N. (1995). Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E. EMBO J. *14*, 5701–5709.

Haney, S.A. (2008). High Content Screening: Science, Techniques, and Applications (Hoboken, N.J.: Wiley-Interscience).

Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidayat, S., Tokunaga, C., Avruch, J., and Yonezawa, K. (2002). Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell *110*, 177–189.

Hizli, A.A., Black, A.R., Pysz, M.A., and Black, J.D. (2006). Protein kinase C alpha signaling inhibits cyclin D1 translation in intestinal epithelial cells. J. Biol. Chem. *281*, 14596–14603, Epub *12006*, 14523.

Hresko, R.C., and Mueckler, M. (2005). mTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes. J. Biol. Chem. *280*, 40406–40416.

Ikenoue, T., Inoki, K., Yang, Q., Zhou, X., and Guan, K.L. (2008). Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. EMBO J. *27*, 1919–1931, Epub 2008 Jun 1919.

Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Ruegg, M.A., Hall, A., and Hall, M.N. (2004). Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat. Cell Biol. *6*, 1122–1128.

Kau, T.R., Schroeder, F., Ramaswamy, S., Wojciechowski, C.L., Zhao, J.J., Roberts, T.M., Clardy, J., Sellers, W.R., and Silver, P.A. (2003). A chemical genetic screen identifies inhibitors of regulated nuclear export of a Forkhead transcription factor in PTEN-deficient tumor cells. Cancer Cell *4*, 463–476.

Kedersha, N., Stoecklin, G., Ayodele, M., Yacono, P., Lykke-Andersen, J., Fritzler, M.J., Scheuner, D., Kaufman, R.J., Golan, D.E., and Anderson, P. (2005). Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. J. Cell Biol. *169*, 871–884.

Kim, J.E., and Chen, J. (2000). Cytoplasmic-nuclear shuttling of FKBP12-rapamycin-associated protein is involved in rapamycin-sensitive signaling and translation initiation. Proc. Natl. Acad. Sci. USA *97*, 14340–14345.

Kim, D.H., Sarbassov, D.D., Ali, S.M., King, J.E., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell *110*, 163–175.

Kimball, S.R., and Horetsky, R.L. (2001). Alterations in interprotein interactions between translation initiation factors assessed by fluorescence resonance energy transfer. Int. J. Biochem. Cell Biol. *33*, 797–806.

Kleijn, M., Scheper, G.C., Wilson, M.L., Tee, A.R., and Proud, C.G. (2002). Localisation and regulation of the eIF4E-binding protein 4E-BP3. FEBS Lett. *532*, 319–323.

Knight, Z.A., Gonzalez, B., Feldman, M.E., Zunder, E.R., Goldenberg, D.D., Williams, O., Loewith, R., Stokoe, D., Balla, A., Toth, B., et al. (2006). A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling. Cell *125*, 733–747.

Konaklieva, M.I., and Plotkin, B.J. (2005). Lactones: generic inhibitors of enzymes? Mini Rev. Med. Chem. *5*, 73–95.

Kowluru, A., Seavey, S.E., Rabaglia, M.E., Nesher, R., and Metz, S.A. (1996). Carboxylmethylation of the catalytic subunit of protein phosphatase 2A in insulin-secreting cells: evidence for functional consequences on enzyme activity and insulin secretion. Endocrinology *137*, 2315–2323.

Lang, V., Zanchin, N.I., Lunsdorf, H., Tuite, M., and McCarthy, J.E. (1994). Initiation factor eIF-4E of *Saccharomyces cerevisiae*. Distribution within the cell, binding to mRNA, and consequences of its overproduction. J. Biol. Chem. *269*, 6117–6123.

Lejbkowicz, F., Goyer, C., Darveau, A., Neron, S., Lemieux, R., and Sonenberg, N. (1992). A fraction of the mRNA 5′ cap-binding protein, eukaryotic initiation factor 4E, localizes to the nucleus. Proc. Natl. Acad. Sci. USA *89*, 9612–9616.

Livingstone, M., Ruan, H., Weiner, J., Clauser, K.R., Strack, P., Jin, S., Williams, A., Greulich, H., Gardner, J., Venere, M., et al. (2005). Valosin-containing protein phosphorylation at Ser784 in response to DNA damage. Cancer Res. *65*, 7533–7540.

Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J.L., Bonenfant, D., Oppliger, W., Jenoe, P., and Hall, M.N. (2002). Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol. Cell *10*, 457–468.

Maira, S.M., Stauffer, F., Brueggen, J., Furet, P., Schnell, C., Fritsch, C., Brachmann, S., Chene, P., De Pover, A., Schoemaker, K., et al. (2008). Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. Mol. Cancer Ther. *7*, 1851–1863.

Mora, A., Komander, D., van Aalten, D.M., and Alessi, D.R. (2004). PDK1, the master regulator of AGC kinase signal transduction. Semin. Cell Dev. Biol. *15*, 161–170.

Oh, N., Kim, K.M., Cho, H., Choe, J., and Kim, Y.K. (2007). Pioneer round of translation occurs during serum starvation. Biochem. Biophys. Res. Commun. *362*, 145–151, Epub 2007 Aug 2008.

Oshiro, N., Yoshino, K., Hidayat, S., Tokunaga, C., Hara, K., Eguchi, S., Avruch, J., and Yonezawa, K. (2004). Dissociation of raptor from mTOR is a mechanism of rapamycin-induced inhibition of mTOR function. Genes Cells *9*, 359–366.

Pause, A., Belsham, G.J., Gingras, A.C., Donze, O., Lin, T.A., Lawrence, J.C., Jr., and Sonenberg, N. (1994). Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5′-cap function. Nature *371*, 762.

Peterson, R.T., and Schreiber, S.L. (1999). Kinase phosphorylation: Keeping it all in the family. Curr. Biol. *9*, R521–R524.

Rong, L., Livingstone, M., Sukarieh, R., Petroulakis, E., Gingras, A.C., Crosby, K., Smith, B., Polakiewicz, R.D., Pelletier, J., Ferraiuolo, M.A., et al. (2008). Control of eIF4E cellular localization by eIF4E-binding proteins, 4E-BPs. RNA *14*, 1318–1327.

Rosen, N., and She, Q.B. (2006). AKT and cancer–is it all mTOR? Cancer Cell *10*, 254–256.

Roux, P.P., Ballif, B.A., Anjum, R., Gygi, S.P., and Blenis, J. (2004). Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. Proc. Natl. Acad. Sci. USA *101*, 13489–13494.

Sabatini, D.M. (2006). mTOR and cancer: insights into a complex relationship. Nat. Rev. Cancer *6*, 729–734, Epub 2006 Aug 2017.

Sarbassov, D.D., Ali, S.M., Kim, D.H., Guertin, D.A., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2004). Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr. Biol. *14*, 1296–1302.

Sarbassov, D.D., Guertin, D.A., Ali, S.M., and Sabatini, D.M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science *307*, 1098–1101.

Smith, I.J., and Dodd, S.L. (2007). Calpain activation causes a proteasome-dependent increase in protein degradation and inhibits the Akt signalling pathway in rat diaphragm muscle. Exp. Physiol. *92*, 561–573.

Tan, E.W., and Rando, R.R. (1992). Identification of an isoprenylated cysteine methyl ester hydrolase activity in bovine rod outer segment membranes. Biochemistry *31*, 5572–5578.

Thoreen, C.C., Kang, S.A., Chang, J.W., Liu, Q., Zhang, J., Gao, Y., Reichling, L.J., Sim, T., Sabatini, D.M., and Gray, N.S. (2009). An ATP-competitive mTOR inhibitor reveals rapamycin-insensitive functions of mTORC1. J. Biol. Chem. *284*, 8023–8032.

Toschi, A., Lee, E., Xu, L., Garcia, A., Gadir, N., and Foster, D.A. (2009). Regulation of mTORC1 and mTORC2 complex assembly by phosphatidic acid: a competition with rapamycin. Mol. Cell. Biol. *29*, 1411–1420.

Vlahos, C.J., Matter, W.F., Hui, K.Y., and Brown, R.F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J. Biol. Chem. *269*, 5241–5248.

Walker, E.H., Pacold, M.E., Perisic, O., Stephens, L., Hawkins, P.T., Wymann, M.P., and Williams, R.L. (2000). Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine. Mol. Cell *6*, 909–919.

Wan, X., Harkavy, B., Shen, N., Grohar, P., and Helman, L.J. (2007). Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism. Oncogene *26*, 1932–1940.

Wang, E., Norred, W.P., Bacon, C.W., Riley, R.T., and Merrill, A.H., Jr. (1991). Inhibition of sphingolipid biosynthesis by fumonisins. Implications for diseases associated with Fusarium moniliforme. J. Biol. Chem. *266*, 14486–14490.

Weinstein, I.B. (2002). Cancer. Addiction to oncogenes–the Achilles heal of cancer. Science *297*, 63–64.

Xu, G.F., O'Connell, P., Viskochil, D., Cawthon, R., Robertson, M., Culver, M., Dunn, D., Stevens, J., Gesteland, R., White, R., et al. (1990). The neurofibromatosis type 1 gene encodes a protein related to GAP. Cell *62*, 599–608.

Yang, J., Shamji, A., Matchacheep, S., and Schreiber, S.L. (2007). Identification of a small-molecule inhibitor of class Ia PI3Ks with cell-based screening. Chem. Biol. *14*, 371–377.

Yu, K., Toral-Barza, L., Shi, C., Zhang, W.G., Lucas, J., Shor, B., Kim, J., Verheijen, J., Curran, K., Malwitz, D.J., et al. (2009). Biochemical, cellular, and in vivo activity of novel ATP-competitive and selective inhibitors of the mammalian target of rapamycin. Cancer Res. *69*, 6232–6240.

Zhang, X., Shu, L., Hosoi, H., Murti, K.G., and Houghton, P.J. (2002). Predominant nuclear localization of mammalian target of rapamycin in normal and malignant cells in culture. J. Biol. Chem. *277*, 28127–28134.