The mammalian target of rapamycin regulates cholesterol biosynthetic gene expression and exhibits a rapamycin-resistant transcriptional profile

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The mammalian target of rapamycin (mTOR) is a central regulator of cell growth and proliferation in response to growth factor and nutrient signaling. Consequently, this kinase is implicated in metabolic diseases including cancer and diabetes, so there is great interest in understanding the complete spectrum of mTOR-regulated networks. mTORC1 exists in two functionally distinct complexes, mTORC1 and mTORC2, and whereas the natural product rapamycin inhibits only a subset of mTORC1 functions, recently developed ATP-competitive mTOR inhibitors have revealed new roles for both complexes. A number of studies have highlighted mTORC1 as a regulator of lipid homeostasis. We show that the ATP-competitive inhibitor PP242, but not rapamycin, significantly down-regulates cholesterol biosynthesis genes in a 4E-BP1-dependent manner in NIH 3T3 cells, whereas S6 kinase 1 is the dominant regulator in hepatocellular carcinoma cells. To identify other rapamycin-resistant transcriptional outputs of mTOR, we compared the expression profiles of NIH 3T3 cells treated with rapamycin versus PP242. PP242 caused 1,666 genes to be differentially expressed whereas rapamycin affected only 88 genes. Our analysis provides a genomewide view of the transcriptional outputs of mTOR signaling that are insensitive to rapamycin.

sterol regulatory element-binding protein-2 | eIF4E-binding protein-1 phosphorylation | 3-hydroxy-3-methylglutaryl-CoA reductase | thioredoxin interacting protein | expression array

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that belongs to the family of PI3K-related kinases. A nodal point in cell signaling, mTOR integrates growth factor signaling with nutrient availability to directly regulate protein translation and affect cell growth and proliferation (1, 2). mTOR hyperactivation caused by mutation or dysregulation of mTOR upstream effectors is frequently observed in human cancers (3, 4). The natural product rapamycin was instrumental in the discovery of mTOR and has provided a pharmacological tool for studying mTOR function in settings in which genetic approaches have been unsuccessful, as mTOR loss of function mutations result in an embryonic lethal phenotype in mice (5). mTOR exists in two distinct complexes, mTOR complex 1 (mTORC1) and complex 2 (mTORC2), of which only the former is sensitive to rapamycin inhibition (6, 7). mTORC1 regulates protein synthesis by phosphorylating both p70 S6 kinase 1 (S6K1) and the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) to control ribosomal protein translation and cap-dependent translation (8). mTORC2 has been less well characterized because of the lack of a specific inhibitor. The only known mTORC2 substrates are Akt and serum- and glucocorticoid-induced kinase (SGK), which it phosphorylates on their C-terminal hydrophobic motifs at Ser473 and Ser422 respectively, to contribute to full kinase activation (9, 10).

Recently developed mTOR inhibitors bind to the active site rather than the rapamycin binding site, thus allowing for pharmacological interrogation of both mTOR complexes. Importantly, active site mTOR inhibitors have also revealed a surprising distinction between the two known mTORC1 substrates, S6K1 and 4E-BP1. Although rapamycin is classified as an mTORC1 inhibitor, it potently inhibits only S6K1 and not 4E-BP1 phosphorylation and cap-dependent translation, whereas ATP-competitive inhibitors PP242, Torin1, Ku-0063794, and WYE-354 completely block both mTORC1 outputs (11–14). The ATP-site inhibitors also completely block cell growth and proliferation in cell lines in which rapamycin has only a partial effect. More recently, phosphoproteomic studies have compared the new generation of mTOR inhibitors with rapamycin (15, 16). In general, ATP-competitive mTOR inhibitors could elucidate new rapamycin-resistant outputs of mTOR.

mTOR signaling has recently been linked to lipid metabolism (17, 18). Fatty acids and cholesterol are not only integral membrane components, but cholesterol and its biosynthetic intermediates serve as precursors for the generation of steroid hormones and other signaling molecules. Moreover, dysregulation of lipid homeostasis is the fundamental basis of disorders such as obesity, diabetes, and cardiovascular disease. Thus, there is great interest in understanding the mechanisms which regulate lipid homeostasis.

Lipid metabolic gene expression is controlled by master transcriptional regulators SREBP-1 and SREBP-2 (sterol regulatory element-binding protein-1 and -2), which, respectively, regulate fatty acid and cholesterol biosynthesis. SREBP proteins are synthesized as precursors that reside in the endoplasmic reticulum membrane, and, when cellular sterol levels are low, SREBP is processed in the Golgi apparatus to release the N-terminal fragment that acts as a transcription factor to control its own expression and the expression of lipid biosynthesis and uptake genes (19, 20). Recent studies that used rapamycin have established a role for mTOR in regulating SREBP-1 processing and the expression of fatty acid biosynthetic genes (21–23). However, conflicting data have been obtained by groups attempting to use rapamycin to study cholesterol biosynthesis. Although activation of Akt, an upstream activator of mTOR, induced the expression of SREBP1 and its target genes, the effect on SREBP2 was less pronounced (24). Moreover, in different cell types, rapamycin has opposing effects on SREBP-2 processing and the expression of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), the rate-limiting step in cholesterol biosynthesis (25–27). Collectively, these data have led to the notion that mTOR may regulate SREBP-1 and SREBP-2 differently and that some regulation may be cell type-specific.

In this study, we use a combination of pharmacological and genetic tools to dissect the molecular basis of mTORC1 regulation of cholesterol biosynthesis. We find that cholesterol bio-

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synthesis is coordinately regulated by 4E-BP1 and S6K1 and the relative contribution of each regulator varies between cell types. Finally, to provide an unbiased assessment of the rapamycin-resistant outputs of mTOR, we carried out genomewide comparative transcriptional profiling of rapamycin and ATP-competitive mTOR inhibitor PP242. Our gene expression profiling revealed a 20-fold increase in the number of PP242-sensitive genes compared with rapamycin. These findings represent a critical step toward our understanding of the complete transcriptional programs regulated through mTOR, which are only mildly or not at all sensitive to rapamycin.

**Results**

Rapamycin-Resistant Role for mTORC1 in Regulating Cholesterol Biosynthetic Gene Expression. In murine NIH 3T3 cells, we confirmed that phosphorysensitive immunoblotting of known mTOR outputs that rapamycin and PP242 inhibit the phosphorylation of ribosomal protein S6 on Ser240/244, but PP242 additionally inhibits mTORC2 phosphorylation of Akt on Ser473 and mTORC1 phosphorylation of 4E-BP1 on Thr37/46 (Fig. 1A). The selected doses of rapamycin and PP242 represent the EC90 values for each drug as measured by antiproliferative activity (11).

We focused on regulation of cholesterol biosynthesis by using PP242 in hopes of identifying a rapamycin-resistant role for mTOR. Quantitative RT-PCR (qRT-PCR) analysis indicated that, in murine NIH 3T3 cells, PP242 was significantly more effective than rapamycin at inhibiting the expression of the transcriptional regulator SREBP2 and its target genes, including HMGCR, the rate-limiting enzyme, and 24-dehydrocholesterol reductase (DHCR24), one of the terminal enzymes in cholesterol biosynthesis (Fig. 1B). Culture in lipoprotein deficient serum (LPDS) did not significantly increase the expression of cholesterol biosynthesis genes in NIH 3T3 cells, so we turned to an LPDS-stimulated HeLa cell system (28). We first validated that, in HeLa cells, culture in LPDS instead of FBS shows significant up-regulation of HMGCR and squalene epoxidase (SQLE), another gene in the cholesterol biosynthetic pathway (Fig. S1). In this LPDS-stimulated cell system, PP242, but not rapamycin, significantly blocks expression of cholesterol biosynthesis genes. We therefore demonstrate that the transcriptional effects of both classes of mTOR inhibitors are consistent between unstimulated and LPDS-stimulated cell systems.

To confirm that the observed transcriptional effects of PP242 were caused by mTOR inhibition and not off-target effects, we also showed that Ku-0063794, a structurally distinct mTOR inhibitor, similarly decreased cholesterol gene expression (Fig. S2). To confirm that PP242 was not a general inhibitor of transcription, we showed that PP242 did not significantly affect ribosomal protein gene RPS16 expression (Fig. S3). We also asked if PP242 regulated the proteolytic processing of SREBP-2; however, SREBP-2 was undetectable by immunoblotting in NIH 3T3 cells.

Because rapamycin is able to effectively block S6K1 activity and not at all sensitive to rapamycin, we hypothesized that these transcriptional outputs were not regulated through S6K1. We took a pharmacological approach to examine the effect of the selective S6K1 inhibitor DG2 on the expression of cholesterol biosynthesis genes. In NIH 3T3 cells, DG2 effectively inhibited ribosomal protein S6 phosphorylation (Fig. 1A) but did not inhibit the expression of SREBP2 and its target genes (Fig. 1B). These results confirmed that cholesterol biosynthetic gene expression was not regulated through S6K1 in NIH 3T3 cells.

To determine which mTOR complex was responsible for the differential gene expression induced by PP242, we used SIN1-knockout (SIN1−/−) primary mouse embryonic fibroblasts (MEFs), which lack a unique and integral component of mTORC2 (29). PP242 down-regulated SREBP2 and HMGCR similarly in the WT and SIN1−/− MEFs, demonstrating that regulation of the cholesterol pathway did not require intact mTORC2 and was therefore controlled by mTORC1 (Fig. 1C).

mTORC1 Effector 4E-BP1 Regulates Expression of SREBP2 and Its Target Genes. A previous study in breast cancer cell lines showed that siRNA knockdown of eukaryotic initiation factor 4E inhibits SREBP1 processing and that expression of a nonphosphorylatable 4E-BP1 mutant inhibits transcriptional activity of a fatty acid biosynthesis reporter gene (23). We asked if 4E-BP1 regulated the expression of SREBP2 and its target genes. We first exploited a cell line that we discovered to be selectively resistant to PP242-mediated inhibition of 4E-BP1 phosphorylation. In SW620 colorectal carcinoma cells, PP242 prevents S6K1 phosphorylation of ribosomal protein S6 at Ser240/244 and mTORC2 phosphorylation of Akt at Ser473, but does not effectively inhibit 4E-BP1 phosphorylation at Thr37/46, whereas in HCT15 colorectal carcinoma cells, PP242 effectively blocks all three mTOR outputs (Fig. 2A). In HCT15 cells PP242 significantly decreases the expression of SREBP2 and target genes HMGCR and DHCR24. Compared with HCT15 cells, SW620 cells express lower basal levels of SREBP2 and DHCR24; nevertheless, HCT15 cells are sensitive to PP242-mediated down-regulation of cholesterol biosynthetic genes whereas SW620 cells are resistant (Fig. 2B). Incomplete resistance to PP242 inhibition of HMGCR and DHCR24 may be the result of a small fraction of unphosphorylated 4E-BP1 in PP242-treated SW620 cells (Fig. 2A). We also tested if overexpression of an unphosphorylatable dominant-negative 4E-BP1 mutant in which all five mTORC1-regulated phosphorylation sites have been mutated to alanine (FLAG-4E-BP1M) could replicate the effect of PP242 on cholesterol gene transcription. NIH 3T3 cells were cotransfected with a tetracycline-inducible FLAG-4E-BP1M expression construct and Tet activating expression construct. Expression of FLAG-4E-BP1M was confirmed by immunoblot analysis, and low levels of 4E-BP1 phosphorylation were indeed detected even in the absence of doxycycline (Fig. 2C). FLAG-4E-BP1M expression caused a decrease in SREBP2, DHCR24, and lanosterol synthase (LS5) gene expression (Fig. 2D). Interestingly, even low levels of FLAG-4E-BP1M expressed in the absence of doxycycline are sufficient to decrease transcript levels of some but not all SREBP2 target genes. As a negative control, FLAG-4E-BP1M overexpression had no significant effect on ACTIN mRNA levels (Fig. S4). In contrast to
our findings in NIH 3T3 cells, FLAG-4E-BP1M expression had no significant effect on cholesterol biosynthetic gene expression in HCT15 and SW620 cells (Fig. S5). We asked if these cell types differed in their endogenous levels of 4E-BP1. We found that NIH 3T3 cells express nearly twice the level of phospho-4E-BP1 as HCT15 and SW620 cells, potentially confounding the effects of the dominant-negative mutant in the latter cell types (Fig. 2E).

**mTORC1 Effector S6K1 Controls SREBP-2 Processing and Cholesterol Biosynthetic Gene Expression in Hepatocellular Carcinoma Cells.** A recent study in MEFs expressing constitutively active mTOR showed that siRNA knockdown of S6K1 causes a decrease in SREBP-1 processing and the expression of fatty acid biosynthetic genes, suggesting that fatty acid biosynthesis is controlled through S6K1 (22). In contrast, studies in retinal pigment epithelial cells and MEFs showed that siRNA knockdown and genetic deletion of S6K1 had no effect on SREBP-1 processing (30). We asked if cell type dictated whether lipid metabolism was regulated mostly through S6K1 or 4E-BP1 and chose to examine hepatocellular carcinoma (HCC) cells because they are derived from the liver and therefore specialized in lipid metabolism.

In HCC cells, PP242 and DG2, but not rapamycin, significantly reduced cholesterol biosynthetic gene expression (Fig. 3A). Although expression of FLAG-4E-BP1M did not affect SREBP2 and its target gene transcript levels (Fig. 3B and C), HCC cells do not contain high levels of phospho-4E-BP1 and thus may not be sensitive to FLAG-4E-BP1M overexpression (Fig. 2E). We next examined the relative amounts of full-length SREBP-2 [SREBP-2 (FL)] and processed SREBP-2 [SREBP-2 (p)] upon inhibitor treatment over a 24-h time course. Phosphospecific immunoblotting confirmed that all three inhibitors effectively block ribosomal protein S6 phosphorylation whereas only PP242 blocks 4E-BP1 phosphorylation. Whereas PP242 and DG2 fully block SREBP-2 (p) formation within 12 h, rapamycin is an incomplete inhibitor (Fig. 3D). The time-dependent disappearance of SREBP-2 (p) correlates with an accumulation of full-length protein. Consistent with previous work that used siRNA knockdown of S6K1 in TSC2-null cells, we suggest that, in HCC cells, S6K1, but not 4E-BP1, is a key regulator of cholesterol biosynthesis (22).

**PP242 but Not Rapamycin Causes Global Transcriptional Rewiring.** Given the differential abilities of rapamycin and PP242 to regulate the cholesterol biosynthetic gene program, we wanted to compare the transcriptional outputs of rapamycin and PP242 in an unbiased manner. Microarray analysis in NIH 3T3 cells revealed that, at a B statistic (log-odds) cutoff of B > 0, PP242 caused 1,666 genes to be differentially expressed whereas rapamycin affected a mere 88 genes (Fig. 4A). Moreover, only 12 of these genes were uniquely rapamycin-sensitive, and for most of the 76 shared targets, PP242 caused a greater magnitude of change in gene expression (Fig. S6). The shared targets included SREBP2 (Srebp2), which was down-regulated 1.4-fold by rapamycin and 1.7-fold by PP242. A previous study had identified more than 500 rapamycin-sensitive genes in human lymphoma cells (31). To address this discrepancy, we filtered our data according to the previously published statistical parameters and observed 236 and 3,892 genes differentially regulated by rapamycin and PP242, respectively, thus bringing our rapamycin analyses in line with previous transcriptional analyses of this drug. Both studies show similar functional classes of genes being targeted by rapamycin inhibition of mTOR. Regardless of the statistical methods used, we see an approximately 20-fold greater number of genes affected by ATP-site inhibition compared with rapamycin inhibition of mTOR.

We next used a more stringent false discovery rate (FDR) cutoff of less than 0.005 to identify a subset of 452 genes that displayed a statistically significant difference in gene expression between PP242 and rapamycin treatment (Table S1). These uniquely PP242-sensitive genes ranged from 6.5-fold down-regulated to 12-fold up-regulated, and we functionally annotated the most strongly up- and down-regulated genes according to the Gene Ontology database (Fig. 4B). The 30 most down-regulated genes were particularly enriched in transcriptional regulation (e.g., E2F transcription factor 2 and early growth response 1) and cell cycle...
control (e.g., cyclin F and aurora kinase A), which is consistent with the ability of PP242, but not rapamycin, to fully inhibit cell growth and proliferation. Genes involved in biosynthetic processes (e.g., thymidine kinase 1) and molecular transport (e.g., importin α2) were also strongly down-regulated. The 30 most up-regulated genes spanned a broad range of functional categories, including catabolic enzymes (e.g., acetyl CoA carboxylase β) and proteins involved in ubiquitin-mediated proteolysis (e.g., cullin 5).

Functional annotation of all 452 PP242-sensitive genes indicated that genes involved in transcription, metabolism, and signal transduction were widely represented in both datasets (Fig. 4C). An overwhelming 35% of down-regulated genes were involved in the cell cycle, which is consistent with the cytostatic effect of PP242. Although genes from all phases of the cell cycle were affected, more than half were involved in mitosis. Transport and DNA damage and repair genes were mostly down-regulated whereas proteolytic, apoptotic, redox, and immune response genes were more prominently up-regulated. Overall, PP242 induces a significantly broader transcriptional response compared with rapamycin, thus providing a rich source of mTOR-regulated pathways previously obscured by the limited effects of rapamycin.

Because PP242 inhibits both mTOR complexes, we expected genes identified in an unbiased screen to be uniquely sensitive to PP242 and not rapamycin to include genes downstream of mTORC2. Thioredoxin interacting protein (TXNIP) was one of the most strongly up-regulated genes, so we assessed its dependence on mTORC2. Txnip is a negative regulator of thioredoxin, and thioredoxin not only maintains cellular redox homeostasis, it also interacts with several transcription factors and signaling molecules including phosphatase and tensin homologue, which signals upstream of Akt and mTOR (32, 33). As a result of the broad cellular roles of thioredoxin, Txnip is implicated in diverse cellular processes including proliferation, apoptosis, and differentiation (34). A recent study identified TXNIP as an upstream regulator of mTOR activity (35), but here we report that mTOR as a regulator of TXNIP. TXNIP was significantly up-regulated by PP242 but not by rapamycin or DG2 (Fig. 4D). In contrast to our results in NIH 3T3 cells, TXNIP was unresponsive to PP242 in WT and SIN1−/−MEFs, so we turned to pharmacological inhibition of mTORC2 substrates as an alternative means to distinguish between mTORC1 and mTORC2 dependence. Inhibition of mTORC2 effectors Akt and SGK by Akt-1/2i and GSK 650394, respectively, did not affect TXNIP expression, suggesting that TXNIP expression is not regulated through mTORC2.

Discussion

The emerging class of ATP-competitive mTOR inhibitors has revealed rapamycin-resistant outputs of mTOR and made pharmacological interrogation of the complete spectrum of mTOR functions possible. Meanwhile, mTORC1 has emerged as a regulator of lipid biosynthesis, although its role in cholesterol metabolism has been less well defined than for fatty acid metabolism as a result of the limited effects of rapamycin. By using ATP-site mTOR inhibitor PP242, we confirmed a role for mTORC1 in cholesterol biosynthesis.

In NIH 3T3 cells, PP242 is much more effective than rapamycin at inhibiting the expression of cholesterol biosynthesis genes, which we attribute to a unique regulatory role of mTORC1 effector 4E-BP1. 4E-BP1 may regulate SREBP-2 activation by controlling the cap-dependent translation of one or more proteins involved in SREBP-2 processing. Alternatively, cholesterol biosynthetic gene transcription is coordinated regulated by SREBP-2 and other transcriptional cofactors, and these auxiliary cofactors may undergo highly cap-dependent translation. Another possibility is that 4E-BP1 regulates the degradation of SREBP2 transcript. It will be interesting to elucidate the exact mechanism by which 4E-BP1 controls transcript levels of SREBP2 and its target genes to control cholesterol metabolism.

mTORC1 regulates not only SREBP2 transcription but also its posttranslational processing. Whereas 4E-BP1 is a primary regulator of cholesterol biosynthetic gene expression in NIH 3T3,
HCT15, and SW620 cells, S6K1 regulated SREBP-2 processing and the expression of its target genes in HCC cells. Interestingly, rapamycin fully inhibited ribosomal protein S6 phosphorylation but was an incomplete inhibitor of SREBP-2 processing, suggesting that SREBP-2 processing is independent of S6 phosphorylation and may be mediated through another S6K1 substrate. Collectively, our data suggest that cholesterol biosynthesis is a complex process that is controlled by multiple mTOR outputs. We propose that S6K1 and 4E-BP1 coordinately regulate cholesterol metabolism and that the relative contribution of each mTORC1 effector varies between cell types.

We hypothesized that there were additional transcriptional consequences of ATP-competitive mTOR inhibition and evaluated this by gene expression profiling in NIH 3T3 cells. Consistent with previous studies showing active site mTOR inhibition to be more effective than rapamycin, S6K1 and 4E-BP1 regulate cholesterol metabolism and that the relative contribution of each mTORC1 effector varies between cell types.

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gene has been associated with an increased risk for lung cancer (39). The broad transcriptional profile revealed by ACT-site
mTOR inhibition offers unique insight into medically relevant pathways controlled through mTOR, and detailed analysis of
this gene set may contribute toward delineating new regulatory networks downstream of mTOR.

Materials and Methods

Inhibitors and Plasmids. Rapamycin, Akt-1/2i, and N-acetyl-leucyl-leucyl-norleucinal (ALLN) were purchased from Calbiochem. PFP124 (40) and DG2 (41) were synthesized as described previously. Ku-0063794 was a gift from Y. Liu and P. Ren (Intellilince, La Jolla, CA) and GK 650394 was provided by D. Pearce (University of California, San Francisco, San Francisco, CA). FLAG-4E-BP1M and rTfA Tet activation constructs were described pre-
viously (42).

Immunoblotting. Anti-SREBP2-2 (1C6) antibody was purchased from BD Biosciences and anti-FLAG (anti-FLAG M2) antibody was from Sigma. All other antibodies were from Cell Signaling Technology. Lysate preparation and immunoblotting are detailed in SI Materials and Methods.

qRT-PCR. Cells were treated with DMSO or inhibitor for 18 h. RNA was harvested, reverse-transcribed, and analyzed by qRT-PCR as described in SI Materials and Methods. All qRT-PCR data were analyzed using one-way ANOVA with multiple comparisons or Student t test when appropriate, and error bars in figures represent the SEM of three biological replicates. Primer sequences are listed in Table S2.

Transfection. Cells were transfection using the Nucleofector transfection system (Amaxa) according to the manufacturer's protocol. Cells (1 × 10⁶) were cotransfected with 5 μg of FLAG-4E-BP1M and 0.5 μg of Tet activator expression constructs. One to 2 d after transfection, cells were induced with 1 μg/mL doxycycline and harvested 24 h later.

Microarray Analysis. NIH 3T3 cells were treated with DMSO, 50 nM rapamycin, or 2 μM PP242 for 18 h. Each treatment was done in triplicate. Sample preparation, labeling, array hybridizations, and statistical analyses are de-
scribed in SI Materials and Methods.

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Supporting Information

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**SI Materials and Methods**

**Cell Culture.** NIH 3T3 and HeLa cells were purchased from American Type Culture Collection. Primary WT and SIN1−/− MEFs were provided by B. Su (Yale University, New Haven, CT). HCT15 and SW620 colorectal carcinoma cells were provided by R. S. Warren (University of California, San Francisco, San Francisco, CA). HCC cells were provided by A. Goga (University of California, San Francisco, San Francisco, CA). NIH 3T3 cells were cultured in DMEM supplemented with 10% BCS and penicillin/streptomycin. WT and SIN1−/− MEFs and HCT15 and SW620 cells were cultured in DMEM containing 10% FBS, glutamine, and penicillin/streptomycin. HCC cells were cultured in Eagle Minimal Essential medium supplemented with 10% FBS and penicillin/streptomycin. HeLa cells were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin. For culture under low-sterol conditions, HeLa cells were grown in media containing 10% LPDS (Intracel) for 24 h, then treated with inhibitor for an additional 18 h and harvested.

**Immunoblotting.** Cells were treated with DMSO or inhibitor for the indicated lengths of time and then lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, Roche protease inhibitor mixture, Roche phosphatase inhibitor mixture). For SREBP-2 immunoblot analysis, cells were harvested. Immunoblots were quantified by using ImageJ software.

**qRTP-PCR.** After DMSO or inhibitor treatment, total RNA was isolated from cells by using QIAshredder and RNeasy Mini kits (Qiagen). RNA (2–5 μg) was reverse-transcribed into cDNA by using SuperScript III Reverse Transcriptase (Invitrogen). Real-time PCR was performed on the StepOnePlus Real-Time PCR System (Applied Biosystems) using SYBR Green Master Mix and the following thermal cycles: 10 min at 95 °C, 42 cycles of 20 s at 95 °C, 30 s at 57 °C, and 30 s at 72 °C. The data were analyzed by the ΔΔCt method, and ribosomal RPL17 was used as an internal control for normalization of transfected NIH 3T3 cells whereas RPL3 was used as a control in all other instances.

**Microarray Analysis.** Sample preparation, labeling, and array hybridizations were performed according to standard protocols from the University of California, San Francisco, Shared Microarray Core Facilities and Agilent Technologies. Total RNA was isolated by using QIAshredder and RNeasy Mini kits (Qiagen). RNA was quantified by Nanodrop and integrity was assessed on an Agilent 2100 Bioanalyzer. RNA was amplified by using whole-transcriptome amplification kits (Sigma) following the manufacturer’s protocol, and subsequent Cy3-CTP labeling was performed by using one-color labeling kits (NimbleGen). The size distribution and quantity of the amplified product was assessed by Nanodrop and Bioanalyzer, and equal amounts of Cy3-labeled target were hybridized to Agilent mouse whole-genome 4x44K Ink-jet arrays. Hybridizations were performed for 14 h, according to the manufacturer’s protocol. Arrays were scanned by using the Agilent microarray scanner, and raw signal intensities were extracted with Feature Extraction software (version 10.1).

The dataset was normalized by using the quantile normalization method (1). No background subtraction was performed, and the median feature pixel intensity was used as the raw signal before normalization. A one-way ANOVA linear model was fit to the comparison to estimate the mean log2-based fold changes and calculate moderated t-statistic, B statistic, FDR, and P value for each gene for the comparison of interest. All procedures were carried out by using functions in the R package limma in Bioconductor (2, 3). Duplicate probes sets for the same gene were eliminated from the final gene list. Data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus database under accession number GSE27784.


**Fig. S1.** mTOR inhibition down-regulates cholesterol biosynthetic gene expression similarly in an unstimulated and an LPDS-stimulated cell system. HeLa cells were cultured in media containing FBS or LPDS for 24 h, then treated with DMSO, 50 nM rapamycin, or 2 μM PP242 for 18 h. Cells were harvested, and mRNA levels were assessed by qRT-PCR (***P < 0.01; ****P < 0.001; ns, not significant).
Fig. S2. ATP-site mTOR inhibitor Ku-0063794 inhibits cholesterol biosynthetic gene expression. Relative amounts of mRNA in NIH 3T3 cells after 18 h treatment with DMSO or 10 μM Ku-0063794 (***P < 0.001).

Fig. S3. PP242 does not affect RPS16 gene expression. Relative amounts of mRNA in NIH 3T3 cells after treatment with DMSO or 2 μM PP242 for 18 h (ns, not significant).

Fig. S4. Expression of FLAG-4E-BP1M does not affect ACTIN mRNA levels. Relative mRNA levels in NIH 3T3 cells transfected with FLAG-4E-BP1M as described in Fig. 2C (ns, not significant).

Fig. S5. Expression of FLAG-4E-BP1M does not affect cholesterol biosynthetic gene expression in SW620 cells. SW620 cells were cotransfected with FLAG-4E-BP1M and Tet activator expression constructs, and induced with 1 μg/mL doxycycline, and cell lysates were prepared for immunoblotting and RNA was harvested for cDNA synthesis and subsequent qRT-PCR analysis. Legend is the same as in Fig. S4. No differences observed by qRT-PCR were statistically significant.
**Functional annotation of the 88 rapamycin-sensitive genes.**

**A** List and functional annotation of the 12 genes that are uniquely rapamycin-sensitive (B > 0).

**B** List and functional annotation of the 76 genes that are differentially affected by rapamycin and PP242 (B > 0).

Legend is the same as in Fig. 4C.
Table S1. Differential gene expression induced by PP242 in NIH 3T3 cells

Table S1

Table S2. Primers used for qRT-PCR analysis

Table S2