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A Genome-wide RNAi Screen for Polypeptides that Alter rpS6 Phosphorylation

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Abstract

Mammalian target of rapamycin (mTOR) is a giant protein kinase that controls cell proliferation, growth, and metabolism. mTOR is regulated by nutrient availability, by mitogens, and by stress, and operates through two independently regulated hetero-oligomeric complexes. We have attempted to identify the cellular components necessary to maintain the activity of mTOR complex 1 (mTORC1), the amino acid-dependent, rapamycin-inhibitable complex, using a whole genome approach involving RNAi-induced depletion of cellular polypeptides. We have used a pancreatic ductal adenocarcinoma (PDAC) cell line, Mia-PaCa for this screen; as with many pancreatic cancers, these cells exhibit constitutive activation of mTORC1. PDAC is the most common form of pancreatic cancer and the 5-year survival rate remains 3–5% despite current nonspecific and targeted therapies. Although rapamycin-related mTOR inhibitors have yet to demonstrate encouraging clinical responses, it is now evident that this class of compounds is capable of only partial mTORC1 inhibition. Identifying previously unappreciated proteins needed for maintenance of mTORC1 activity may provide new targets and lead to the development of beneficial therapies for pancreatic cancer.

Keywords

rpS6; Phosphorylation; mTOR; Genome wide; RNAi; Screen; Immunofluorescence; Pancreatic cancer

The phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin-pathway (PI3K/AKT/mTOR-pathway) regulates cell proliferation, cell survival, angiogenesis, and resistance to antitumor treatments. In tumors, the PI3K/AKT/mTOR-pathway is activated through several different mechanisms, most commonly activating mutations in PI-3 kinase or loss of PTEN expression. mTOR functions through two independently regulated complexes, both of which are activated by PI-3 kinase (1). The mechanisms underlying activation of mTOR complex 2 by PI-3 kinase, as well as the contribution of mTORC2 to the malignant phenotype are poorly understood. In contrast, based primarily on extensive work using rapamycin and its congeners, activated mTOR complex 1 is thought to support several aspects of the malignant phenotype; therefore, inhibition of mTORC1 is widely considered a promising antitumor treatment (2–7). The pathway from PI-3 kinase to TOR is well described, proceeding through Akt inhibition of the Tuberous Sclerosis complex, a GTPase activating protein for the ras-like GTPase Rheb,

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³Expand and freeze down as many vials as possible originating from the same lot and the same passage. It is advisable to use a fresh vial for each transfection so that all experiments are performed with cells corresponding to the same passage. This lowers the probability that mutations will accumulate while the cells are maintained in culture and prevent alterations in cell growth rate over time. In addition, this also facilitates performing the experiment at a defined schedule and allows each experiment to be independent of others. The cell growth rate of each experiment remains unaffected.

¹⁰Using the small bore wellmate cartridge will lead to a loss of approximately 100–150 µl of the final working antibody solution.

and Rheb activation of mTOR complex 1 (8). Independently, mTORC1 activity is also regulated by cellular amino acid levels such that depletion of intracellular amino acids, most notably leucine (9, 10), will inactivate mTORC1 despite the presence of a highly active PI-3 kinase pathway. The cellular elements that comprise this amino acid-dependent regulatory input are much less well known, save for the Rag GTPases, however, apart from their ability to bind raptor and thus modify mTORC1 subcellular localization, the regulation of the Rag GTPases is largely obscure (11). We undertook a genome-wide RNAi screen to uncover previously unknown components needed to sustain mTORC1 signaling, using pancreatic cancer cells. In addition to providing a more comprehensive understanding of this signaling pathway, the identification of polypeptides and pathways necessary for mTORC1 activity in such cells may identify “druggable” targets whose inhibition may complement and/or augment the efficacy of the direct mTOR and PI-3 kinase inhibitors currently available or in development (12).

We chose ribosomal S6 phosphorylation as the indicator of mTORC1 signaling activity. S6 is the single phosphopeptide of the mammalian 40S ribosomal subunit (13). Ribosomal S6 is phosphorylated *in vivo* on five serine residues (235, 236, 240, 244, and 247) located within the carboxylterminal 15 amino acids of the S6 polypeptide (14). Phosphorylation at each of these sites is catalyzed in a sequential, processive reaction (236 first, then 235, 240, 244, 247) by the p70S6 kinase/S6K1 (15). In support of the suitability of S6P to serve as a reporter of mTORC1 activity is the ability of rapamycin to inhibit S6 phosphorylation and S6K1 activity in essentially all mammalian cells examined. Thus, although S6 can also be phosphorylated by the Rsk family of kinases, which appear to sustain S6 phosphorylation in S6K1/2 deficient mice (16), Rsks are insensitive to rapamycin and appear to play no part in S6 phosphorylation in normal or malignant cells (17). S6K1 is a direct substrate of the mTOR complex 1, which phosphorylates several sites in an autoinhibitory region in the carboxylterminal tail, and most importantly, Thr389/412 in a hydrophobic motif just carboxylterminal to the S6K1 catalytic domain (18); the latter phosphorylation creates a binding site for the protein kinase PDK1, which then phosphorylates Thr229/252 in the S6K1 activation loop (19). Together, the phosphorylations at Thr389/412 and Thr229/252 activate S6K1 in a synergistic manner (19). The critical importance of Thr389/412 phosphorylation in S6K1 activation makes mTORC1 essential for S6K1 activity. The phosphorylation of S6K1(Thr389/412) is completely inhibited by rapamycin (IC₅₀~ 2 nM) in all cells (20).

Inasmuch as S6K1(Thr389/412-P) is a direct indicator of mTORC1 activity, we first examined the feasibility of using this modification as a readout for a high-throughput assay using antibody-based immunofluorescence (IF) detection. Although this phosphorylation is a reliable indicator of mTORC1 activity in a western blot format (20), conversion to high-throughput formats was not feasible. This is so because of the very low abundance of endogenous S6K1 allows the small background of nonspecific fluorescence associated with all S6K1(Thr389/412-P) antibodies in this mode of detection to swamp the specific signal generated from the low abundance S6K1(Thr389/412-P).

In contrast, ribosomes are highly abundant cellular constituents, and especially so in transformed cells. Although the physiological/functional significance of S6 phosphorylation remains obscure (21), ribosomal S6 protein is unquestionably the best characterized, most specific and abundant S6K1 substrate. Recent studies with rapamycin and the more potent mTOR catalytic site inhibitors have shown that a considerably higher mTORC1 activity is required to maintain S6K1(Thr389/412) phosphorylation than is needed to sustain 4E-BP(Thr37/46) phosphorylation (22); thus the loss of the S6K1 substrate, S6P provides a very sensitive indication of the inhibition of mTORC1. After screening a variety of monoclonal and polyclonal phospho-specific S6 antibodies, we selected a monoclonal antibody directed

at S6(Ser235P/236P) and optimized the conditions necessary for detection of pS6 by IF that provide maximum sensitivity and minimal background signals in a high-throughput fashion (i.e., 384-well plate).

The following criteria are important in choosing a cell line: transfection efficiency, reproducibility across experiments, dynamic range (signal to background ratio), and z' score (see below). Seeking to favor identification of positive regulators of mTOR complex 1, we chose a cell line with a high level of mTORC1 activity, as reflected by the level of rpS6 phosphorylation (see below) under usual culture conditions, thereby obviating the need for a stimulation of the cells prior to analysis, and providing the largest possible dynamic range between the initial and inhibited levels of S6P.

The Mia-Paca 2 cells are considered a representative pancreatic cancer cell line based on their mutational profile (i.e., K-Ras mutant, P53 mutant, P16HD, and DPC4 wild-type) (23, 24). We chose them for this screen because the mTOR/PS6K/PS6 pathway is constitutively active (25), because the cells are reasonably transfectable, and because their morphology enabled reliable quantitation of the IF signals on a cell by cell basis.

Immunofluorescence-based HTP screens can be accomplished using either high content microscopes or plate readers with fluorescent capability. Plate readers, which sum fluorescence from an entire well or segment, may facilitate a higher throughput, but at the cost of cell-specific information, e.g., subcellular localization, cell cycle stage, etc., as well as an ability to focus detection on specific cell populations or subcellular compartments. After testing different plate readers (Envision, LICOR Aeries, M5) and reading methods (top and bottom read), we decided that high content microscopy, using the IXM apparatus, enabled analysis on a cell-by-cell basis and provided the best possible signal-to-background ratio. Only high content microscopy provides assurance that the IF signal in fact originates from the target, minimizing false positives.

The z' -score – used for assessment of assay conditions and for optimization of HTP screens – is given by the formula:

$$z' = 1 - \frac{(3SD_{+control} + 3SD_{-control})}{(AVG_{+control} - AVG_{-control})}$$

The dynamic range, i.e., the difference among the positive and negative controls and the variance of the measurements are thus reflected by the z' , providing a useful measure of overall assay performance.

The smart pool PLK1 oligo is used routinely to monitor transfection efficiency; knockdown of PLK1 gene expression induces a G2/M arrest and gives a cell-death phenotype. Five different commercially available transfection reagents and different ratios of the transfection reagent (amount of transfection reagent used) to the input RNAi (25, 50, 100 nM), as well as the duration of exposure to the transfection mix were evaluated. The reproducibility across experiments is monitored by the z' . Transfection efficiency proved to be strongly dependent on cell confluency/cell number at plating, with approximately 50% confluency proving optimal. At cell densities significantly above this level, transfection efficiency fell, perhaps due to competition for RNAi molecules, even at the practical upper limit of 100 nM. Moreover, imaging becomes problematic at very high cell numbers. At lower cell numbers, the abundance of cells appropriate for imaging may be insufficient even after optimizing transfection efficiency. As regards termination of the experiment, several timepoints (48, 72, and 96 h) posttransfection should be evaluated.

In selecting optimal conditions, the following were most important: z' -score (which incorporates the fold difference among positive and negative RNAi controls as well as the dynamic range of the signal), the ease and accuracy with which a cell line can be imaged (adherent spread-out cells are more easily imaged as compared to others), and whether a cell line behaved consistently in terms of its cell growth rate, the intensity of the primary readout, i.e., PS6 and the transfection efficiency.

2. Materials

2.1. Cell Culture

1. Dulbecco's Modified Eagle's Medium (Gibco/BRL, Bethesda, MD) supplemented with 10% fetal bovine serum (FBS), Hyclone (Hyclone Laboratories, Logan, Utah) (see Note 1), horse serum (Gibco/BRL), and penicillin/streptomycin.
2. Trypsin (0.05%) EDTA (1×) (0.53 mM EDTA in HBSS).
3. Countess® Cell Counting Chamber Slides (Invitrogen, Carlsbad, CA).
4. PCR Mycoplasma test: MycoSensor PCR Assay Kit (Stratagene, Santa Clara, CA).

2.2. Transfection

1. Whole si-genome SMARTpool RNAi library (Dharmacon, Lafayette, CO): Targets the whole genome and is consisted of RNAis against 21,176 genes. Each RNAis is a pool of four individual double-stranded RNAi oligonucleotides, mixed together.
2. Controls:
 - Non-Targeting siGenome SMARTpool.
 - FRAP1 siGENOME SMARTpool.
 - PLK1 siGenome SMARTpool.
3. Transfection reagent (empirically determined, using the cell line of choice): Lipofectamine 2000 (Invitrogen, Carlsbad, CA).
4. Opti-MEM (GIBCO/BRL, Bethesda, MD).
5. Nuclease-free water (Qiagen, Valencia, CA).
6. 5× siRNA Buffer (Dharmacon, Lafayette, CO).
7. Two types of tubing cartridges: Eight-channel standard bore disposable tubing cartridge (presterilized) and small bore disposable tubing cartridge (Thermo Scientific, Hudson, NH).
8. Aspirator Tool – “The Wand”: It is a 24-channel adaptor and is used for aspirating liquid from 384-well assay plates. Drummond Scientific stainless steel wand (VWR, West Chester, PA).
9. 30 µL 384-tip racks, nonsterile (Velocity11).
10. RNAase Zap Wipes (Ambion, Austin, TX); RNAase Zap (Ambion, Austin, TX).
11. 384-Well plates Assay Plates (Corning Incorporate, Corning, NY).
12. Bleach-Rite. BRSPRAY16 (Current Technologies Inc., Crawfordsville, IN).

¹It is important to keep the same FBS lot the same through the screen. Different lots have differential effect on cell growth rate which in turn affects the PS6 readout. If there is need to change serum lots, then you should initially test the number of cells plated per well and adjust this number as a function of the optimal PS6.

13. Sharpie: Silver Metallic 39100 (website: <http://www.sharpie.com/>; available at Staples).
14. Instant Sealing Sterilization Pouches (7 × 13 in.). These are autoclave bags in which a matrix wellmate cartridge can be sterilized (Fisher Scientific, Pittsburg, PA).

2.3. Immunofluorescence

1. PBS (containing CaCl₂ and MgCl₂; GIBCO, BRL). Alternatively, prepare 10× PBS stock solution by the addition of 8 g NaCl, 0.2 g KCl, 1.15 g Na₂HPO₄ (sodium phosphate, dibasic), 0.2 g KH₂PO₄ (potassium phosphate, monobasic). Add water to 1 l. Adjust pH to 7.4.
2. Formaldehyde (37% solution).
3. Methanol.
4. Block solution (Containing 1% Goat serum and 5% Horse Serum). Goat Serum.
5. Primary Antibodies:

S6: Phospho-S6 Ribosomal protein (Ser 235/236) Rabbit monoclonal Antibody (Cell Signaling Technology). For longer term storage the antibody should be aliquoted and stored at −80°C. Avoid freeze–thaw of the aliquots. 4000 is a clone 2F9 4854 but produced recombinantly.

Phospho-S6 Ribosomal Protein (Ser235/236) (2F9) Rabbit mAb (Cell Signaling Technology).

Total S6S6 Ribosomal Protein (54D2) Mouse mAb (Cell Signaling Technology).

- 6 Secondary antibody: Alexa Fluor® 488 goat anti-rabbit IgG (H + L) highly cross-adsorbed; 2 mg/mL (Invitrogen, Carlsbad, CA).
- 7 Nuclear stain: DAPI (Sigma, St. Louis, MI). Make stock solution in water and then dilute 1:1,000.
- 8 Cover for plates: Thermowell Sealing Tape (Costar Corning Incorporate, Corning, NY).

3. Methods

3.1. Transfection

1. For all transfection and immunofluorescence steps: Use a Matrix-Wellmate (ThermoFisher) to dispense all cells and for all subsequent liquid dispensing steps. The following steps are all performed in a TC hood (Class II Biohazard). Please refer to Notes 2–⁴ for comments on TC methods.
2. Thoroughly wipe the hood using 70% ethanol and kim-wipes. If needed preclean with either 10% bleach or Bleach-Rite. Anything that is placed in the hood should be cleaned with 70% ethanol. It is advisable that pipettes are also cleaned with RNAase zap and then rinsed with water.

²Follow standard mammalian tissue culture guidelines (29).

⁴Test the cells for mycoplasma either by PCR or by DAPI staining; many mycoplasma PCR kits are commercially available. Mycoplasma can cause the cell responses to drift over time and is one of the causes of unreproducible results.

3. Transfer of RNAs from the library stock plates to assay plates: The Velocity 11 Bravo automated liquid handling platform is used to transfer 4 μ l of 1 μ M RNA from the library plates to the assay plate by use of the Velocity 11 384-well tips. The final RNAi concentration is 100 nM. This type of transfection is referred to as “dry,” in that the RNA is added first to the plate and the transfection reagent in Opti-MEM (referred to as “reagent mix”) is added thereafter. Alternatively, a “wet” transfection is one in which case the transfection reagent mixed with the Opti-MEM is added first to the assay plates followed by the RNAi oligonucleotides.
4. Label assay plates (Corning) using a silver pen (Shapiro).
5. The Wellmate small bore tubing cartridge, used to dispense small volumes of liquid (i.e., the transfection reagent mix and subsequently the cells), is used for all subsequent dispensing steps during the transfection. Before using the cartridge, rinse with nuclease-free water (3 $\times$), 70% ethanol (1 $\times$) followed by nuclease-free water (3 $\times$). Make sure that the cartridge is not clogged and that all liquid is removed by priming with air. Ensure sterility of the cartridge by autoclaving it. It can be autoclaved up to three times according to the manufacturer. Use a test plate to determine whether the alignment or the height of the Wellmate requires adjustment. Examine visually whether the liquid is dispensed onto the center of the well; this is necessary in order to ensure homogeneous addition of the cells and the transfection reagent mix. Adjust the height of the dispensing head by raising or lowering it to the required height. Adjust the x-stage position of the wellmate if necessary, following the user manual instructions.
6. Add Controls: The RNAi smart pool oligonucleotides are shipped in dry form. Resuspend the oligo in 1 $\times$ RNAi Buffer to 100 μ M and then to 1 μ M. Prepare 1 $\times$ buffer by diluting the 5 $\times$ Buffer with nuclease-free water. Add controls to as many wells as possible (at least 16 wells for each control per plate).
7. Prewarm media, Opti-MEM and trypsin in a 37°C tissue culture – dedicated water bath before use. Prepare transfection reagent with medium: In a 50-ml sterile falcon tube mix, prepare a mix containing Lipofectamine 2000 with Opti-MEM at a ratio of 1:100 (e.g., for 20 ml of final volume use 200 μ l of Lipofectamine 2000) (see Note 5). Incubate the transfection reagent mix at room temperature for 5 min. During this time, invert the tube containing the transfection reagent mix a couple of times and spin at 233 $\times g$ for 1 min. Repeat twice to ensure the transfection reagent mix is equally mixed (see Note 6). Set the dispensing volume of the wellmate to 10 μ l and add the transfection reagent mix to each well. Be sure the tubing cartridge is devoid of bubbles and use the highest dispensing speed (S1) for dispensing the mix. After the transfection mix has been added subject the plates immediately to centrifugation at 233 $\times g$ for 1 min. It is preferable that the temperature of the centrifuge is set at room temperature.
8. Prepare cells: The cells should be passaged twice before the day of the transfection. The confluency of the cells at the time they are split is very critical as this will in turn affect the cell growth rate during the course of the experiment. At the time of harvest for transfection, cells should be approximately 80–90% confluent. A cell count is always used to determine cell number at the time of transfection.
9. Trypsinize cells. Remove media and wash off residual serum with trypsin–HBSS (see Note 7). Prepare media to be used for the transfection omitting the antibiotics.

⁵For Lipofectamine 2000: Keep Lipofectamine 2000 on ice and/or minimize the time that Lipofectamine is left at room temperature, inasmuch the exposure of Lipofectamine 2000 to nonoptimal temperatures can affect efficiency of the reagent.

⁶We have observed empirically that further incubation of the transfection reagent mix decreases transfection efficiency.

After addition of media containing 10% FBS to neutralize trypsin, spin down cells at $335 \times g$ for 5 min in a 50-ml sterile tube. Remove the supernatant and resuspend the cell pellet in transfection media. Pipette up and down the cell pellet so that you have a homogeneous cell population. Count the cells by using a hemacytometer or an automated cell counter (as available). To ensure that the cell number is accurate, it is recommended that you assay four replicates. Prepare the final dilution of the cells: 95 cells/ μ l plated per well ($\times 26 \mu$ l) or 2,500 cells/well. Please note that this number is dependent on the serum lot/catalog number since different serum lots can differentially affect the cell growth rate and therefore the cell number needs to be optimized accordingly. Use a 250-ml flask to make the final dilution from which cells are dispensed. Since the cells will settle quickly, it is critical to swirl the flask continuously while dispensing to ensure there will be a homogeneous distribution of the cells. Use a test plate first to visually inspect that the dilution is accurate. You should aim for a plating confluency of around 50–55%. A denser seeding density will lead to increased cell number which in turn will cause the cells to starve and decrease PS6 baseline levels (see Note 8). At 2 h after addition of cells to the transfection mix, spin down plates for 1 min at $233 \times g$. Use the aspirator wand to remove media. To prevent dislodging of the cells during all the aspiration steps, place tape around the edges of the wand. The wand can be autoclaved to ensure sterility. Before and after each use it is best to use distilled water so as to prevent clogging of the wand and deposition of particles. Decontaminate the wand with 70% ethanol. Next, add 70 μ l of transfection media per well using the small-bore wellmate cartridge. Change the dispensing speed to S2 so as to prevent the cells from being dislodged. Spin down the plates for 1 min at $233 \times g$. Place in a 37°C tissue culture incubator for 72 h. During this time interval, the growth rate of the cells can be inspected visually.

10. Edge effects are a concern as they alter the outer wells; their occurrence is primarily dependent on the humidity and configuration of the incubator. Edge effects are usually evident from the loss of volume from outer wells or by diminished cell viability in those wells. If the outer two columns and rows contain experimental samples the following approaches can be taken to minimize edge effects. (a) 48-h posttransfection add 10–15 μ l of transfection media to the outer well and columns, (b) place moist paper towels in a container and place the assay plates on top of the paper towels.

3.2. Immunofluorescence

1. Harvest the cells 72 h after beginning the transfection (see Notes 9–¹¹).
2. First wash the cells once with $1 \times$ PBS (Cat. No. 11490).

⁷Be careful to not allow trypsin to remain on cells longer than needed as this will decrease the baseline P-S6 signal.

⁸Try to work as fast as possible. Mia-Paca 2 cells will start clumping after 50–60 min post-trypsinization.

⁹Before harvest, inspect visually the phenotype of the cells in the control wells. Take of the confluency in wells transfected with a non-specific RNAi targeting oligo. If the confluency is less than approximately 85%, delay the harvest for a few more hours. For all steps during the IF protocol other than the two antibody addition steps, use the white (large) bore Wellmate cartridge and the S3 dispensing speed. Inasmuch as the Mia-Paca 2 cells are composed of an adherent epithelial cell population and a globular, more loosely attached population, all steps are preceded by a 2-min centrifugation step at $233 \times g$, which serves to preserve both cell populations. Similarly the S3 dispensing speed rate minimizes shear stress imposed on the cells, minimizing loss of the loosely attached cells during the course of the experiment. Liquid is removed using the aspirator wand. Note the strength of the vacuum and the residual volume throughout the course of the experiment, seeking to keep these uniform. If the vacuum strength is weak, increase the number of washes based on the residual volume. With a residual volume of 10–15 μ l, five washes each after fixation and after permeabilization step are sufficient.

¹¹The remaining primary antibody working dilution that remains in the cartridge can be recycled and used within a period of 15 days.

3. Fix the cells for 15 min at room temperature in 4% formaldehyde. Prepare a 4% formaldehyde solution fresh each time and dilute in PBS from the 37% formaldehyde stock. Note the plate order to which formaldehyde is added. When aspirating the fixation buffer keep the same order so that all plates are fixed for the same amount of time. Wash, Spin at $233 \times g$, and aspirate using the wand: this step is repeated five times.
4. Permeabilize the cells by addition of ice-cold methanol for 10 min. Keep the methanol at -80°C or -20°C for a short time prior to addition, to ensure that it is cold when added to the cells. Subsequent to permeabilization repeat the steps noted above: wash, spin, aspirate five times.
5. Prepare the blocking solution composed of 5% HS and 1% GS in PBS. Incubate with blocking solution for 1 h at room temperature.
6. Prepare the working antibody dilution at a final concentration of $2.185 \mu\text{g/ml}$ in block solution in a 50-ml falcon tube. Ensure that antibody is evenly mixed by inverting the tube several times and subsequent centrifugation at $233 \times g$ for 1 min. Repeat this mix/spin step six times. Alternatively, you can prepare the working final antibody dilution and place it on a platform shaker for 30 min prior to adding the antibody.
7. Centrifuge plates ($233 \times g$ for 1 min). Antibody addition can be performed in one of the following two ways: (a) Aspirate the wash solution using the aspirator wand. Remove the residual volume using a fine tip (attached to a vacuum) from the edge of each well. This step needs to be performed rapidly (5 min or less for the whole 384-well plate) so that the cells do not dry out. If more time is needed, then adjust accordingly the number of wells that you process at a time. The antibody can be added either manually using a multiwell pipette or by using the small bore wellmate cartridge and the S2 dispensation rate. Add $15 \mu\text{l}$ of the working antibody dilution per well. Spin down plates. Cover plates with seals (see Note 5). (b) Alternatively, the removal of the residual volume can be avoided; if a residual volume of $10\text{--}15 \mu\text{l/well}$ remains, use an antibody solution of $4.38 \mu\text{g/ml}$ (twice the above final concentration) and add $15 \mu\text{l}$ using the small bore Wellmate cartridge. Centrifuge plates and then cover with sealing tape. In practice, we found this method to give higher background and less well-defined staining (see Note 6). The primary antibody can be stored at 4°C for 1 month; for a longer storage times, aliquot and store the antibody at -80°C . Avoid freeze-thaw cycles of the antibody.
8. Incubate antibody-loaded plates overnight at 4°C for 16–20 h using a shaking platform at low speed.
9. Centrifuge plates and aspirate the primary antibody using the wand. Wash five times with PBS, spin down the plates, aspirate using the wand.
10. Before use, spin the secondary antibody in a microcentrifuge for 20 min at top speed and take care to use only the supernatant so as to eliminate addition of formed protein aggregates to your working solution; precipitated antibody will cause nonspecific staining. For this reason, the highly cross-absorbed secondary antibody is preferred over other types (see Note 12).

¹²If you plan to use the secondary antibody within 1 month, the antibody can be stored at 4°C in the dark. For a longer storage, prepare single-use aliquots and store at -20°C in the dark. The manufacturer claims that the antibody is stable for up to 6 months at -20°C .

11. Prepare the secondary working antibody dilution (Alexa 488; 1:200 dilution of stock) in block solution at a final concentration of 0.01 mg/ml as described above for the final dilution of the primary antibody.
12. Addition of the secondary antibody: Add 15 μ l of the working secondary antibody dilution per well using the small bore cartridge and the S2 dispensation rate. Spin down the plates. Perform these steps involving the secondary antibody in the dark if possible and/or cover the plate with an aluminum seal cover. Cover plates with foil. Incubate for 2 h at room temperature in the dark.
13. Removal of secondary antibody: Spin down plates and aspirate the secondary antibody with the aspirator wand. Wash five times as above using the large bore cartridge and the S3 dispensing rate.
14. Addition of the nuclear dye: Dissolve DAPI in water (20 mg/ml). Aliquot and store at -20°C in the dark. For a DAPI working solution, add 20 μ l of the DAPI stock per ml of D-PBS (1:1,000 dilution) and add 80 μ l/well; incubate for 10 min at room temperature. Take note of the order of the plates to which DAPI was added. Remove DAPI from the plates using the wand in the same order. Wash three times with PBS as described above (wash, spin, aspirate). Seal the plates with sealing tape. The plates are now ready to be imaged (Fig. 1).

The washing steps can also be performed using a plate washer after determining settings that allow retention of both cell populations and the total cell number.

Secondary assays are performed with the goal of identifying where validated/confirmed hits map within the pathway or for functional analysis (e.g., cell death, cell proliferation). For example, one can choose TSC1/2 null MEFs in order to delineate whether positive hits act downstream of the TSC1/2 complex. Using new cell lines, one needs to optimize the transfection conditions as described above. Additionally, other target readouts are needed to determine whether a hit acts through TORC1 or TORC2.

3.3. Image Acquisition

1. For image acquisition, run samples on the High Content Imaging microscope ImageXpress® Micro System (Molecular Devices). Configure and save the “Acquisition” settings that will be used for all subsequent experiments. Binning refers to the camera binning one binning is equal to one pixel; two binning combines a 2 by 2 set of pixels, three binning a 3 by 3 set of pixel, etc. Choose one binning which allows maximal resolution. Gain refers to the amplification applied to the camera signal, with “1” giving least gain and least background amplification; however at a binning of 1 $\times$, a gain of 2 is required to enable sufficient signal (Fig. 2a, screenshot).
2. Select the objective magnification at which the images will be acquired (10 $\times$ Plan Fluor 0.3 NA objective).
3. Run the LAF (Laser AutoFocus) wizard by following the steps described so that the plate parameters are defined. The LAF Wizard defines the multiple settings required for each specific plate type and a particular objective so that the objective will move to an appropriate position under the well and plate bottom, so that the cells will be in focus for image acquisition (Fig. 2a, lower screenshots). It is important that the plate parameters (plate bottom, bottom thickness, etc.) are accurately defined. If the plate parameters are not accurate, out-of-focus images result.

4. Choose the number of sites per well you wish to image. This is dependent on the total number of cells you would like to have imaged. We used four sites/well, however, up to 81 sites may be chosen; alternatively one can choose to image a fixed number of cells per well. A minimum of 1,200 cells/well are needed for this protocol.
5. Inasmuch as only one image plane is sought (i.e., not time-lapse images) “one” time point is selected; alternatively a Z-stack acquisition journal may be used to select the optimal image plane, in which case the time points reflect the number of stacks used.
6. Choose the number of wavelengths that you will image at (in this case, DAPI, FITC).
7. Once the plate settings have been configured, define the focus by choosing one of the following three options to acquire images.
 - a. Laser-based: Select a sample to be imaged and use the “Find Sample” option to adjust focus (Fig. 2b). For each wavelength, adjust the offsets from the z plane at which images are acquired. One can choose the well-bottom autofocus option (runs faster) or both the plate and well bottom option (takes longer but is more accurate), when variation of the Z height in the well exceeds the plate thickness; this is typically the case in thin-bottomed (<150 μm) plastic plates. The Mia-Paca 2 cells are not positioned on the single z plane because they are composed of two cell populations, adherent and globular. If laser-based focusing is not sufficient to enable one to observe a well-defined staining, choose either one of the following:
 - b. Laser plus Image-recovery: if the laser fails to find the image in some wells, an autofocus program will define an appropriate image (Fig. 2c).
 - c. Journal-based: this will allow a z-stack at different planes (Fig. 2d). You can use the “acquire z stack” and define the number of planes you need to acquire as well as the step size (e.g., if the number of planes is 10 and the step size 1 μm , this means that ten images 1 μm apart will be acquired). The software will retain the image with the best focus as the final image. Alternate methods for image acquisition at different z planes also exist. This last option is preferred when the cells grow at different planes. A z-stack allows a well-defined acquisition of images and is particularly useful when the cells’ morphology is more globular than epithelial.
8. Once the settings have been saved you are ready to run the plates. First examine at a couple of different exposure times the control wells, those in which cells are transfected with RNAi against TOR and a nonspecific RNAi. The purpose is to obtain the exposure time that corresponds to the best z’. Always inspect images obtained from Control wells visually to assess staining quality. After all plates have been run, use the cell scoring module for analysis (described in detail below). The primary readout for identifying hits is % positive cells.

3.4. Image Analysis

1. Images are analyzed and quantified by using the MetaXpress® imaging software. Use the cell scoring application module to detect, count and document measurements from the primary FITC readout. The specific module can be applied to the nucleus, cytoplasm, or both; this assay focused on cytoplasmic staining. The cell scoring module (a) identifies nuclei and segments them by using the nuclear

DAPI marker, and (b) identifies positive cells according to a FITC (i.e., S6P) fluorescence intensity that exceeds a predetermined value, i.e., one that provides the highest signal-to-background/noise ratio, as well as the percent positive cells, i.e., the number of FITC positive cells/total number of DAPI positive cells (Fig. 3).

2. In configuring the analysis settings you choose the preview option to inspect and alter these as needed. To do so, go to “review plate data,” select an image and open it. Define the minimum and max width for both the DAPI and FITC readouts which corresponds to the minimum and max width of the nuclei in the case of DAPI and of the cytoplasm in the case of FITC. You can manually count the number of nuclei from representative images ($n = 10$) by using the option “manually count objects.” Then you can set different minimum and maximum widths and choose those that most accurately describe the average number of nuclei you obtained manually. For both readouts, define the intensity above background, i.e., the threshold above which cells will be considered positive. A threshold of FITC fluorescence to score a cell “positive” should be one that provides a signal-to-background ratio at least above three for cells that appear positive by visual inspection, although the dynamic range of images obtained from cells transfected with mTOR RNAi versus nonspecific RNAi may be tenfold or more.
3. Select the specific measurements you wish to collect, here most essential are total cell number (as measured by DAPI), number of positive and/or percent positive cells as defined by the primary FITC readout. General procedures for collecting these values are described in the MetaXpress® 3.1 Analysis guide.

The following measurements may be used as secondary readouts:

Positive cells, total cell number/per site, total area of positive cells, mean area of positive cells.

Positive cells Integrated Intensity: By using the cell scoring module, you consider all positive cells for which the intensity is above a certain threshold. However, in the case of the Mia-Paca 2 cells (and possibly other cells) there are several cell populations of different FITC intensities, e.g., high, medium, and low. This parameter can be used to classify the primary hits according to the sum of the intensity. Alternatively, you can use the Multi-Wavelength Scoring Module to classify the cell populations and determine the number of cells that fall in each category.

Importantly, perform the screen in triplicate to confirm reproducibility of individual candidate genes, to reduce the number of false positives due to off-target effects, and to increase the robustness and confidence as to which are the potential positive hits. Select as “cherry picks” candidate genes that show a consistent response in at least two of the triplicates (i.e., either an increase or decrease of the basal P-S6 levels), and rescreen using the four deconvoluted siRNAs from each pool separately at 100 nM concentration.

3.5. Data Analysis

1. Exclude replicates in which the average total cell number was 2 SD below the average total cell number corresponding to the mTOR positive control; this eliminates proliferative failure for reasons other than inhibition of mTOR.
2. Obtain a normalized S6P value for each well: Divide the absolute number of cells scored as positive for S6P by the total cell number so as to obtain the percentage of positive cells corresponding to each transfected RNAi, i.e., “% positive cells.”

3. Confirm visually the “% positive cells” values from the mTOR and the nonspecific targeting (NS1) RNAi controls. The correspondence between the visual images and the calculated “% positive cells” values reflect the data quality of each experiment.
4. To identify hits, use a level of “% positive cells” that is 2 or preferably 3 SD above or below that of the average value of “% positive cells” in all wells on the plate transfected with a scrambled RNAi, minimally 16 wells/plate. For this calculation, convert “% positive cells” values to z -scores that correspond to each value/hit by using the Excel program. The z score is obtained by the formula:

$$z = \frac{X - \text{AVG}}{y * \text{SD}},$$

where X = “% positive cells” value corresponding to the specific well, AVG = average value obtained from all wells on the plate transfected with a scrambled RNAi, y = the number of SD chosen, i.e., 2 or 3, * means “times.”

5. To obtain the primary hits, define the z score which you will use as a cut-off beyond which to identify hits. Most published screens use a z score of ± 2 as a cut-off. This criterion is user-dependent and fairly arbitrary.
6. Exclude any wells with out-of-focus images, which are obvious by visual inspection (DAPI stain) and also evident when the average total cell number is discordant among the three plates.
7. For all of the wells for which the z was above the cut-off value ($z = \pm 2$), calculate the probability corresponding to each z by using the formula (in excel):

$$Q = (1 - x) * 100$$

where x = Absolute value $(1 - [1 - \text{NormalDistribution}(z)] * 2)$ (26) Subsequently, calculate the cumulative probability considering all three assay plates that corresponds to a specific hit by taking the average of all probabilities, since the events are independent. The strength of each RNAi, i.e., strong, medium, or weak is judged by the average Q of all replicates. You can define these ranges of by comparison with the values observed for known positive and negative mTOR regulators.

Sample calculation:

Example: % positive cells value for TOR = 4.5%

AVG % positive cells value for NS = 67.6%

SD corresponding to NS = 9.8%

% positive value for a specific hit in three replicates = 9.8, 7.6, 8.1

$$z = (67.6 - 9.8) / (3 * 9.15) = 2.10;$$

$$z = (67.6 - 7.6) / (3 * 9.15) = 2.18;$$

$$z = (67.6 - 8.1) / (3 * 9.15) = 2.16$$

$$y1 = \text{ABS}(1 - (1 - \text{NORMSDIST}(B19)) * 2) = 0.96; y2 = 0.97; y3 = 0.97$$

$$1 - y = 1 - \text{ABS}(1 - (1 - \text{NORMSDIST}(B19)) * 2) = 1 - 0.96 = 0.04$$

$$Q1 = 100 \cdot (1 - y) = 4\%; Q2 = 3\%; Q3 = 3\%$$

Average $Q = 3.19$

- 8 Rank RNAi SMART pools in order of average Q ; similarly rank deconvoluted RNAi oligos in order of average Q . Decide on the average Q values that will define hits as strong, medium, and weak. In constructing your final list of primary hits, it is practical to exclude LOCs, hypothetical and retracted/retired genes.

3.6. Validation of Hits Obtained from the Primary Screen

3.7. Data Summarization

1. Whereas in the primary screen, a pool of four RNAi oligos are used with a total concentration of 100 nM, validation uses the individual RNAis at 100 nM; otherwise methods are as in steps 3.1–3.7 above.
1. Analysis of RNAi screening data, use the spotfire software program licensed through TIBCO Software Inc. <http://www.tibco.com> website; Spotfire® DecisionSite® 9.1.1 for Lead Discovery (TIBCO, Palo Alto, CA). It provides data summarization, data visualization, and clustering. This program is user friendly allowing graphing and data tabulation by drag-and-drop actions. Alternatively, software programs from Acuity Software, Inc. are also suitable.

This process is best visualized while using the software, the following description will be most easily absorbed in that manner.

- 2 First create a worksheet in excel containing all the data you want. For example, it can contain the following parameters: % positive cells for replicates A, B, C, z-score for plate A/B/C, individual and average probability, total cell number, library plate number, well number. Import the data into Spotfire. A scatter plot is generated in the main graphing area. You can change the X and Y axis by dragging and dropping the different fields from the filter panel onto the x/y axis.
- 3 An easy and fast way to create charts, scatter plots, and bar graphs containing all data parameters is to use the Spotfire “HTS guide,” which can automatically generate a variety of graphs. The guide creates visualizations to review your HTS data. To run the guide, first create a unique identifier which will represent the library plate number and the well number. To do so, select the “new column from expression” under Data and enter an expression in the window whose syntax will be accepted. To create a column based on plate and well ID you can enter, e.g., STOCK ID&WELL. This identifier is referred to as “calculated column.” Select as a type of review to analyze, type “single run.” Go to: Guides > Data Analysis > Review HTS data > pick single run > pick a results column. Select the parameter you want to have displayed on the results column. Among the choices are average % positive cells A/B/C, average % positive cells of all the triplicates, z-score for plates A/B/C, average z score created from the average % positive cells of all triplicates, average Q . Select how the plate position will be identified in your data set. Identify row and column position (A1, A2). The row and column data can be shown in the same column. The results column can be any of the above fields (imported from Excel: z factor for plate A/B/C), % average for plate A/B/C. Select the sample ID column: Stock ID. Select as your plate ID the calculated column. Select same column for well positions. Choose well and click to create columns. This will lead to creation of six visualizations that will popup. You can select to view each one individually or all simultaneously. You can select the fields that you need to have included in

the analysis: Examples include % positive cells, z , cumulative Q . The following visualizations will be displayed: pie plate sum by result: displays size by taking the sum of the average of % positive cells, results by standard deviation; two examples are shown in Fig. 4. The following are some of the program's applications in the context of the screen:

One can determine the degree of reproducibility between the plates by comparing the standard deviations from the mean for any particular normalized value among the triplicate plates (i.e., between replicate plates A vs. B, B vs. C, A vs. C). One can directly compare the % positive cells value and z scores between replicate plates A vs. B, B vs. C, A vs. C. One has the option of including data from all wells: experimental, positive and negative controls, empty wells or one can filter out the wells you wish to exclude (e.g., one can select only experimental wells and thus deselect all other types under the Filter Options Query Devices). One can select different colors to represent different library plates. Similarly, one can construct other graphs with all relevant fields. One can use the “details on demand” to examine a subset of data points in more detail. Similarly, one can select to visualize results from one or more library plates.

3.8. Bioinformatics/Computational Analysis

1. It is usually desirable to carry out a classification of hits into biological processes, molecular function, and categories by analyzing the validated hits using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Mountain View, CA; <http://www.ingenuity.com>) and Genego (St. Joseph, MI; <http://www.genego.com>). Both IPA and Genego are web-based software programs that use scientific information extracted from journal articles, textbooks, and other data sources to construct canonical pathways and functional categories (27, 28). These programs help define what is known about a hit or set of hits; the main questions that may be addressed through such computational analyses are the putative identities of the signaling, metabolic, and other functional categories represented in the hit list, what pathways are most prominently represented.
2. Genego: Create a list in excel containing the confirmed hits (i.e., those positive with two or more of the individual RNAs of your pool). Include the gene name, or the entrez gene ID or any of the other relevant gene identifiers recognized and listed as options, as well as the average probability that corresponds considering all replicates. Upload your experimental data using the EZ Start window so as to generate an experimental data set that is selectable (an “active” data set) for all subsequent analyses.
3. One can use the “Functional Enrichment by Ontology” function to perform ontology enrichment of the validated hit list for a selected ontology. The Functional Ontology Enrichment tool, which maps the hits to the built-in ontology, is comprised of GeneGo Maps (which represent biochemical or signaling pathways), network ontologies, and some gene ontologies (including processes, molecular functions, and localizations). The “Enrichment by Protein Function” tool can be used to determine the number of objects that belong to different protein classes (e.g., receptors, ligands, proteases, kinases, transcription factors, phosphatases, etc.). This tool uses the p -value to sort results; the p -value corresponds to the probability to have a given value of r , where r = number of network objects from the hit list for a given protein class (e.g., kinases). You can choose the default 5% false discovery rate (FDR) filter as the cut-off or a more stringent one. This program will generate a list of the maximum $-\log(p\text{-value})$ for every term, a value that corresponds to the probability a specific gene set has occurred by chance based on its size (defined as the number of hits found present in the gene set). Stated

differently, p -value is the probability of a match between the dataset and a specific ontology occurs by chance, given the size of the database. A low p -value indicates a larger number of hits belonging to a specific process/pathway and the higher the probability that there is a match between a group of hits and the ontology found.

4. Perform a “pathway analysis” to determine all the interactions associated with the validated hits and whether such interactions are part a known pathway.
5. Perform a “workflow data analysis report” with the dataset. Use the hit list to build networks of pathways and processes associated with the validated hits and sort pathways for importance by the G -score, which is based on the number of Canonical Pathways used to build the network. A network with a high G -score indicates that it contains many Canonical Pathways (i.e., set of genes that participate in a canonical biological process). Another option – referenced under the “data analysis workflows” – is to Perform an Enrichment Analysis which scores and ranks the most relevant cellular processes, disease targets, biomarkers, toxicity processes, and molecular functions for the hit list. Detailed specific information is available online at the Genego site.
6. Ingenuity Pathway Analysis (IPA) uses a knowledge database (the Ingenuity Pathways Knowledge Base) that contains information about genes, chemicals, cellular and disease processes, signaling, and metabolic pathways to identify the biological functions, canonical pathways, and networks that are most significant to the validated hit list. This curated database is comprised of protein interaction networks derived from published protein–protein interactions and/or associations, including direct and indirect protein interactions (such as those involved in physical binding, enzyme–substrate relationships, and transcriptional control). In IPA, networks are comprised of pathway network nodes (which are your hits/proteins) and edges (which are the biological relationship between the hits). First, create an Excel file containing the validated hit list that includes the gene name, Entrez Gene ID, accession number, and average probability corresponding to the z' from all three replicates.
7. Generate protein interaction networks, i.e., a dataset containing the validated hits (=H) by uploading into IPA the excel file containing the gene identification names. Compare the hits against a global molecular network derived from the information in the Ingenuity Knowledge Base (IKB). Generate networks of these hits algorithmically by including hits and other proteins from the IKB which enable the generation of a network based on connectivity. Identify canonical pathways and functions from the IPA library based on their significance to the dataset (hits). To generate the network(s) from the hit list and to define which networks interact with specific hits, upload the excel file containing the gene IDs into IPA, and ensure that each hit is uniquely and accurately recognized by the software. Protein interaction networks are then generated automatically and ranked by “score,” which is the negative log of this probability estimate (p -value) and defined as $\text{score} = -\log_{10}(p\text{-value})$. To generate hypothetical protein networks you can include direct and/or indirect protein interactions, include all or specific species, tissues, and cell lines. The hits are compared against the IPA database. The significance of the association between the hit list and a canonical pathway is measured by the ratio of the number of hits that map to the pathway divided by the total number of proteins that exist in the canonical pathway and by the p -value that is obtained by comparing the number of hits to the total number of genes/proteins in all pathway annotations stored in the IPA database. A low p -value suggests that the association between the hits and these pathways is significant and not random. A p -value $p \leq 0.05$ is considered statistically significant.

8. Perform a biomarker analysis to identify potential biomarker candidates.
9. You can also create/visualize new pathways which originate from the data set that correspond to the hit list, add novel (newly identified) components to already known pathways and/or identify already existing canonical pathways not previously associated with a specific disease or pathway.

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Fig. 1.

Immunofluorescence analysis (IF) of rpS6Ser(235/236) phosphorylation. Mia-Paca 2 cells in 384-well plates were transfected with a control, nonspecific RNAis NS1 (*upper panel*) and (NS2) (*lower panel*) (**a**), S6K, TOR, and Raptor (**b**), TSC1, TSC2, and PTEN (**c**). The cells were then left unperturbed in 10% media for 72 h. Subsequently, they were fixed, permeabilized, and stained by using a rabbit monoclonal anti-PS6 primary antibody. Shown here are representative images; PS6 antibody (Ser 235/236) in green detected with secondary anti-rabbit Alexa 488 antibody. Nuclei are stained with DAPI. Samples were run on the High Content Imaging microscope ImageXpress® Micro and analyzed using the MetaXpress Imaging Software.

a

b

Fig 2. Screenshots depicting the image acquisition settings as described in the main text. **(a)** Setup of general settings as described in the Image acquisition section. **(b–d)** Setup of specific settings: **(b)** Laser-based settings. **(c)** Image-based settings. **(d)** Use of a journal that allows image acquisition at different z heights.

Fig 3.

A screenshot of the cell scoring module for analysis of the acquired images. Shown on the left (*column 1*) is the positive control (cells transfected with RNAi for mTOR) and on the right (*column 3*) is the negative control (cells transfected with a nonspecific targeting RNAi oligo). To the right of each set (*columns 2 and 4*) are the segmentation data; shown in *green* and *red* are the cells that are scored as positive when the threshold intensity is set at 100 and 500 for the DAPI and the primary FITC readout, respectively.

Fig. 4.

Examples of results obtained by use of the spotfire program. **(a)** The z-score corresponding to each well for a total of three library plates containing different RNAi oligos. Each plate is depicted in a different color. **(b)** A representative scatter plot demonstrating the degree of reproducibility among two replicate plates containing identical RNAis; one can calculate the correlation coefficient, which ranged from ~ 0.67 to >0.9 . Candidate genes that show consistent response in two of the three replicates (i.e., either an increase or decrease of the basal P-S6 levels) are selected as potential positives, and rescreened using each of the four individual siRNAs from each pool.