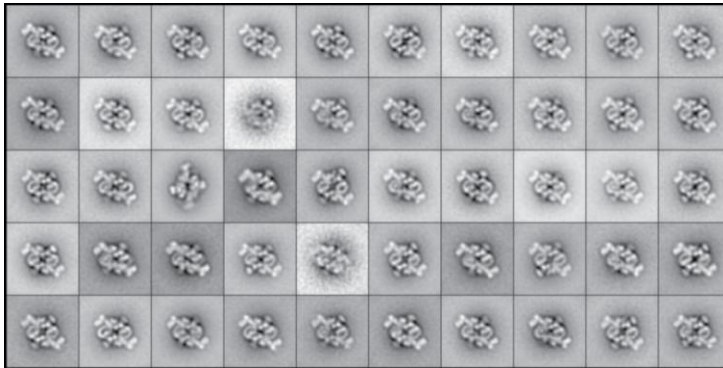


Supplemental Information**Figure S1****Figure S1, related to Figure 1. Class averages of negatively stained mTORC1.**

The 50 class averages were obtained from classification of 10,800 particle images of negatively stained mTORC1. The side length of each panel is 45 nm.

Figure S2

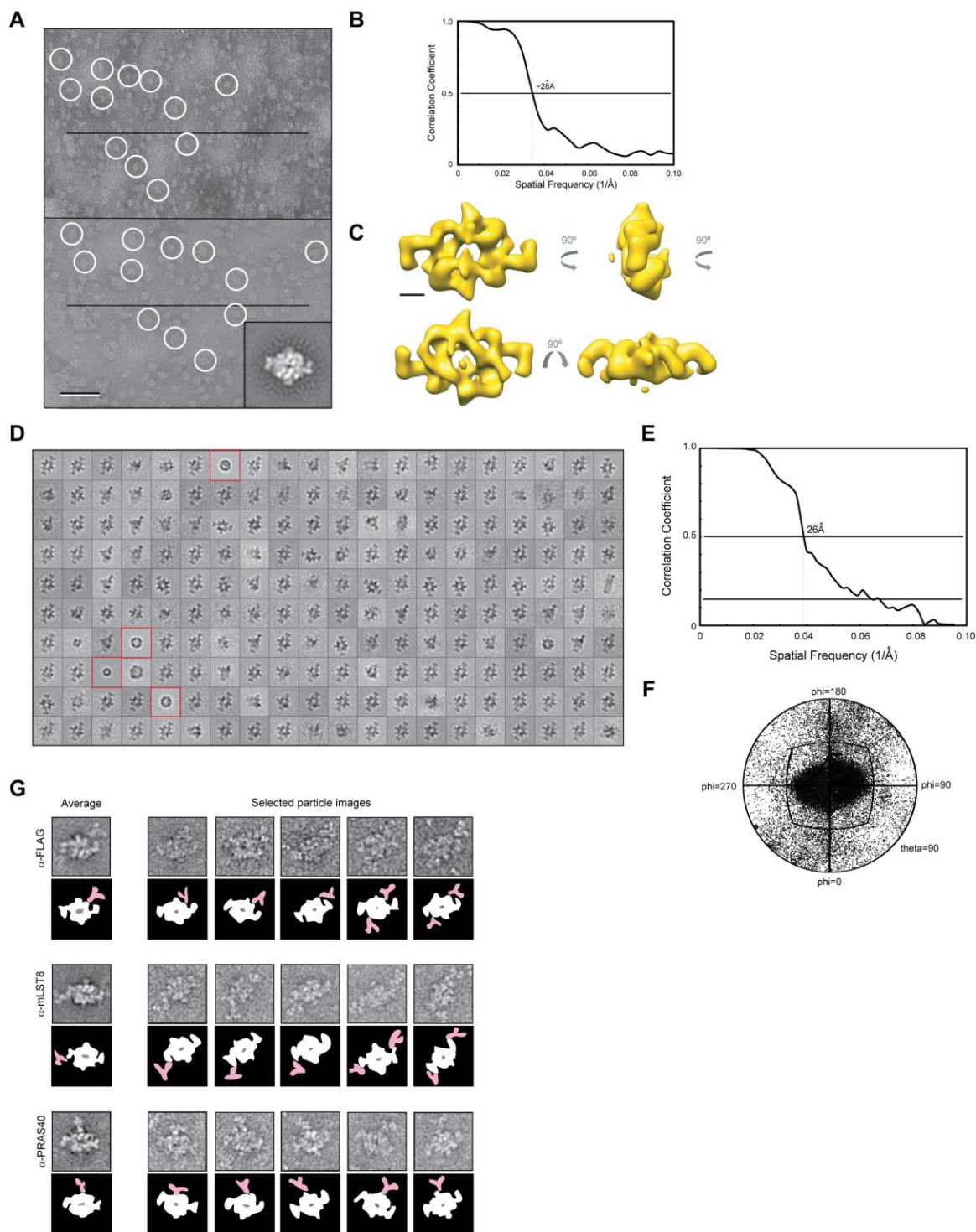


Figure S2, related to Figure 2. 3D reconstruction and subunit organization of mTORC1.

(A) Tilt pair of cryo-negatively stained mTORC1 particles at 0° (*bottom*) and 50° tilt (*top*). The line in each image indicates the tilt axis. The scale bar represents 50 nm. The inset shows a representative class average from the classification of 8,679 particles. The side length of this panel is 54 nm.

(B) Fourier shell correlation (FSC) curve of the 3D reconstruction. The resolution of the density map is 28 Å according to the FSC = 0.5 criterion.

(C) 3D reconstruction of cryo-negatively stained mTORC1. Different views of the reconstruction are shown rotated by 90° about the vertical and horizontal axes as indicated by the arrows. The scale bar represents 5 nm.

(D) Class averages of vitrified mTORC1. The 200 class averages were obtained from classification of 30,494 particles. The side length of each panel is 42 nm. The red squares indicate CCT chaperonin particles, major contaminants in the mTORC1 samples that were removed for subsequent image analysis.

(E) FSC curve of the cryo-EM reconstruction of mTORC1. The resolution of the density map is 26 Å according to the FSC = 0.5 criterion.

(F) The angular orientations of vitrified mTORC1 particles. Plot of the Euler angles for the 28,325 particles used in the 3D reconstruction and refinement in FREALIGN, showing that the complex adopts preferred orientations.

(G) Antibody labeling of mTORC1. A representative class average (*left*) and galleries of individual antibody-labeled particles (*right*) are shown. The schematic representation below each image shows the position of the bound antibody in pale red. Double-labeled particles were occasionally observed in the labeling experiments with anti-FLAG and anti-mLST8 antibodies. The side length of each panel is 45 nm.

Figure S3

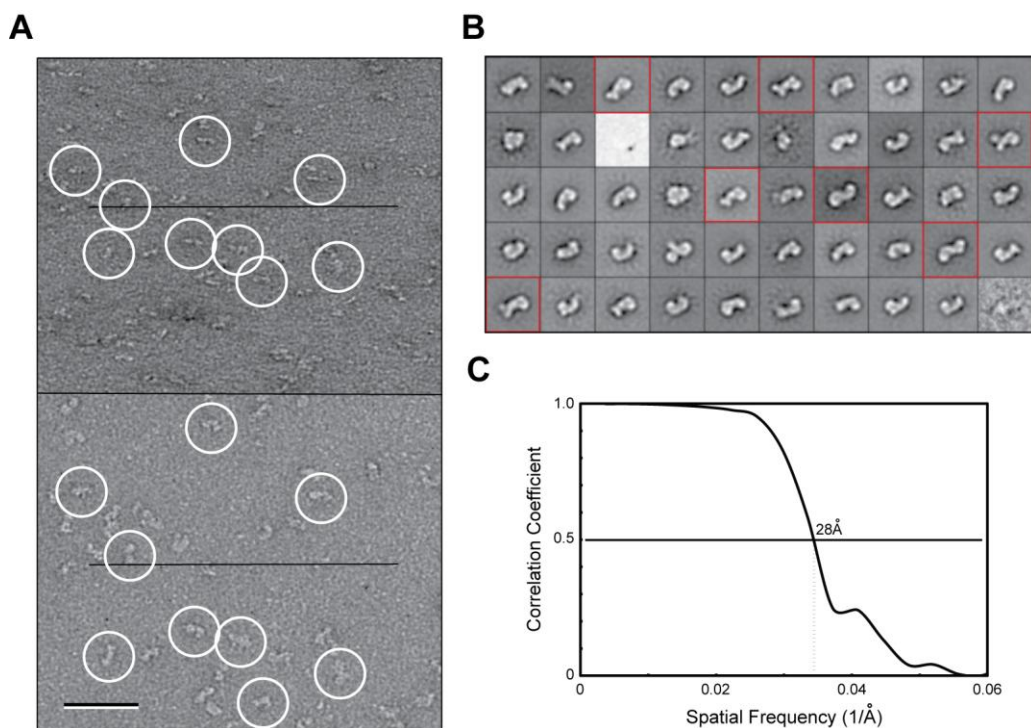


Figure S3, related to Figure 3. 3D reconstruction of negatively stained raptor.

(A) Tilt pair of negatively stained raptor particles at 0° (*bottom*) and 60° tilt (*top*). The line in each image indicates the tilt axis. The scale bar represents 50 nm.

(B) Class averages of negatively stained raptor. The 50 class averages were obtained from the classification of 12,216 particles of negatively stained raptor selected from the images of the untilted sample. The side length of each panel is 27 nm. The red boxes highlight averages with an additional density likely to represent bound PRAS40.

(C) FSC curve of the raptor reconstruction. The resolution of the density map is 28 Å according to the FSC = 0.5 criterion.

Figure S4

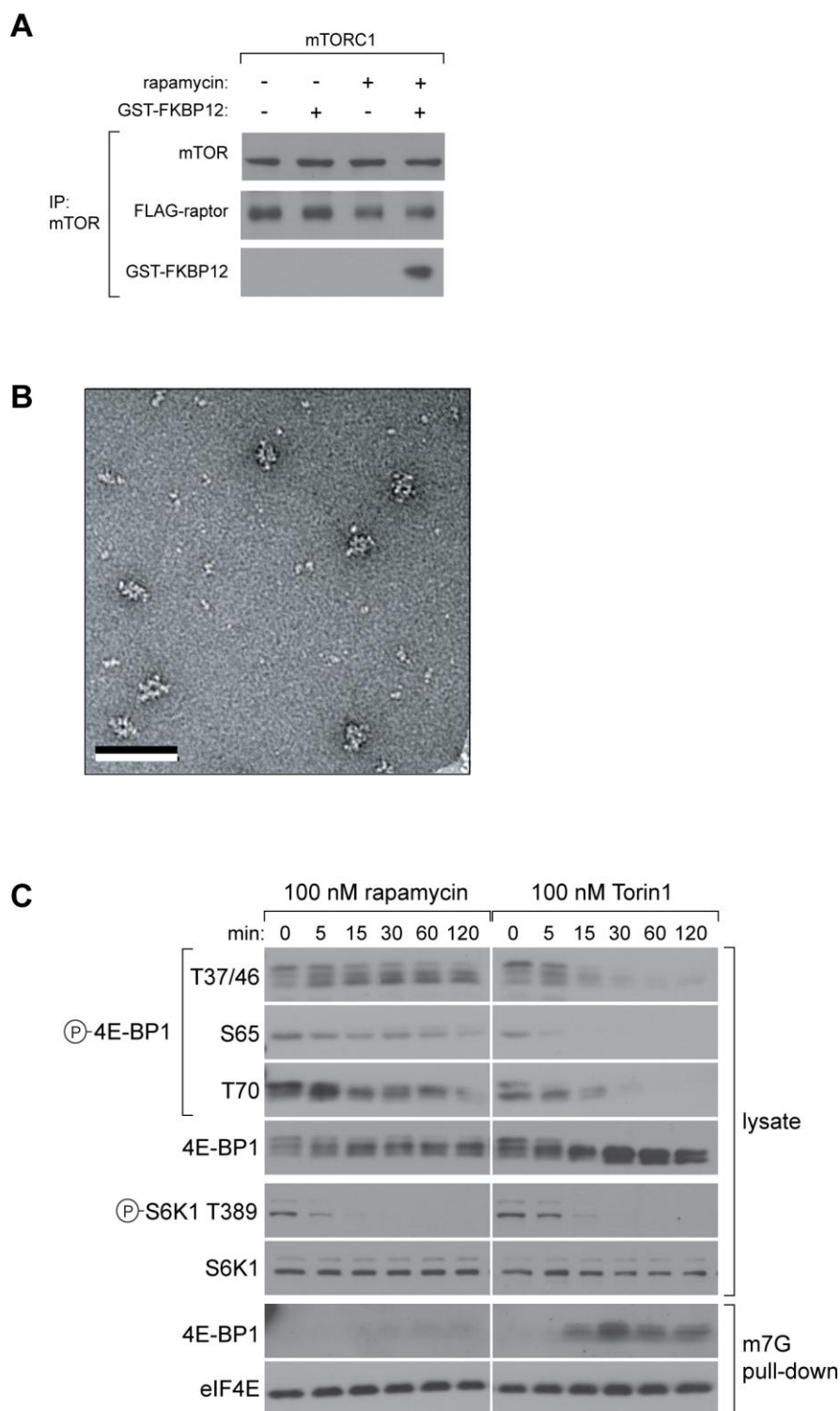


Figure S4, related to Figure 4. Effects of Rapamycin-FKBP12 on mTORC1.

(A) mTORC1 pulldown assay with GST-FKBP12-rapamycin. Purified mTORC1 was treated with 100 nM rapamycin and 0.02 $\mu\text{g}/\mu\text{l}$ GST-FKBP12 for 15 min and co-immunoprecipitated using the mTOR antibody (Santa Cruz). Samples were subsequently analyzed by SDS-PAGE and immunoblotting.

(B) Rapamycin-FKBP12 binding to mTORC1. EM image of negatively stained mTORC1 treated with FKBP12-rapamycin for 15 min.

(C) Time-course study of rapamycin and Torin1 treatment *in vivo*. HEK-293T cells were treated with 100 nM of rapamycin or Torin1 for the specified amount of time, and cell lysates were analyzed by immunoblotting for the levels of the indicated proteins and phosphorylation states. Additionally, eIF-4E was purified from lysates using 7-methyl-GTP-Sepharose and analyzed by immunoblotting.

Supplemental Experimental Procedures

Materials

Reagents were obtained from the following sources: antibodies to phospho-Thr-389 S6K, phospho-Thr-36/47 4E-BP1, phospho-Ser-65 4E-BP1, phospho-Thr-70 4E-BP1, 4E-BP1, mTOR, raptor, mLST8 and eIF-4E from Cell Signaling Technology; antibodies to mTOR (immunoprecipitation), and horseradish peroxidase-labeled anti-mouse, anti-goat, and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; purified recombinant 4E-BP1 from Stratagene; Protein G Sepharose from Thermo Scientific; FuGENE 6 and Complete Protease Mixture from Roche Applied Science; FLAG M2 antibody, FLAG M2-agarose, and ATP from Sigma; Superose 6 10/300 GL and 7-methyl-GTP-Sepharose from GE Healthcare; rapamycin from LC Laboratories; Dulbecco's modified Eagle's medium from SAFC Biosciences; inactivated fetal calf serum, SimplyBlue Coomassie G, and SilverQuest Staining kit from Invitrogen. Torin1 was synthesized and purified by Nathanael Gray (Harvard Medical School) (Thoreen et al., 2009).

Protein expression and purification

To produce soluble mTORC1, we generated a HEK-293T cell line that stably expresses N-terminally FLAG-tagged raptor using vesicular stomatitis virus G-pseudotyped MSCV retrovirus. mTORC1 was purified by lysing cells in 50 mM HEPES, pH 7.4, 150 mM NaCl and 0.4% CHAPS. Cells were lysed at 4°C for 30 min, and the insoluble fraction was removed by centrifugation at 18,000 rpm for 30 min. Supernatants were incubated with FLAG-M2 monoclonal antibody-agarose for 1 hr and then washed with two column volumes of wash buffer 1 (50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM DTT and 2 mM ATP and 0.1% CHAPS) and another two column volumes of wash buffer 2 (50 mM HEPES, pH 7.4, 200 mM NaCl, and 0.1% CHAPS). Purified mTORC1 was eluted with 100 µg/ml 3x FLAG peptide in 50 mM HEPES, pH 7.4, 500 mM NaCl and 0.1% CHAPS. Eluted fractions were pooled and concentrated by centrifugation prior to gel filtration. Protein was further purified by a tandem Superose 6 10/300 GL column (GE Healthcare) in 50 mM HEPES, pH 7.4 and 150 mM NaCl on an AKTA purifier (GE Healthcare).

***In vitro* kinase assay**

HEK-293T cells rinsed once with ice-cold PBS were lysed in ice-cold lysis buffer (40 mM HEPES, pH 7.4, 2 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 0.3% CHAPS or 1% Triton X-100, and one tablet of EDTA-free protease inhibitors per 25 ml). The soluble fractions of cell lysates were isolated by centrifugation at 13,000 rpm for 10 min in a microcentrifuge. For immunoprecipitation of mTOR, primary antibody (Santa Cruz) was added to the lysates and incubated with rotation for 1 hr at 4°C. A 50% slurry of protein G Sepharose was then added, and the incubation continued for an additional 1 hr. Immunoprecipitate was washed three times each with low salt wash buffer (40 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 0.3% CHAPS or 1% Triton X-100). Recombinant S6K1 was purified as described previously (Sancak et al., 2007). Kinase assays were performed for 5 min at 30°C in a final volume of 20 µl consisting of the kinase buffer (25 mM HEPES, pH 7.4, 50 mM KCl, 10 mM MgCl₂, 500 µM ATP) and 150 ng of inactive 4E-BP1 or S6K1 as a substrate. Reactions were stopped by the addition of 60 µl of sample buffer and boiled for 5 min. Samples were subsequently analyzed by SDS-PAGE and immunoblotting.

Negative staining and 2D analysis

For conventional negative staining, samples were adsorbed to glow-discharged carbon-coated copper grids, and stained with 0.75% (w/v) uranyl formate as described (Ohi et al., 2004). Images were collected with a Tecnai T12 electron microscope (FEI) operated at 120 kV. Images were recorded on imaging plates under low-dose conditions at a nominal magnification of 67,000x and a defocus value of -1.5 µm. Imaging plates were read out with a Ditabis Micron imaging plate scanner (DITABIS Digital Biomedical Imaging System AG, Pforzheim, Germany) using a step size of 15 µm, a gain setting of 20,000 and a laser power setting of 30%. 2 x 2 pixels were averaged to yield a pixel size of 4.5 Å on the specimen level. Individual particles were interactively selected from the raw images using Boxer, the display program associated with the EMAN software package (Ludtke et al., 1999). The particles were windowed into 100 x 100 pixel images,

rotationally and translationally aligned, and subjected to 10 cycles of multi-reference alignment using SPIDER (Frank et al., 1996). Each round of multireference alignment was followed by K-means classification. The references used for the first multireference alignment were randomly chosen from the raw images.

Antibody labeling

Purified mTORC1 was incubated with anti-FLAG (Sigma), or anti-mLST8 (Cell Signaling Technology), or anti-PRAS40 (Millipore) at dilutions of 1:15 to 1:50, for 15 to 30 minutes in buffer (50 mM HEPES, pH 7.4, 150 mM NaCl) at room temperature. The samples were negatively stained and imaged as described above. 300 to 800 labeled particles were selected for each of the three labeling experiments and subjected to alignment and classification as described above, specifying 50 output classes.

Rapamycin-FKBP12 binding

For purification of GST-FKBP12, *Escherichia coli* BL21 (DE3) was transformed with the expression construct pGEX4T-FKBP12 grown to an OD₆₀₀ of ~0.6 at 37°C in LB broth containing 100 mg/ml ampicillin and induced with 0.5 mM IPTG. Cells were harvested after a 3-hr incubation at 37°C, resuspended in buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 4 mM DTT), sonicated, and centrifuged at 30,000 x g for 35 minutes. Extract was incubated with Immobilized Glutathione (Pierce). The resin was washed, and bound proteins were eluted with lysis buffer containing 50 mM reduced glutathione. GST-FKBP12 was further purified by gel filtration chromatography using a Superose-12 column (GE Healthcare) on an AKTA purifier (GE Healthcare). 0.02 µg/µl of GST-FKBP12 with 50 nM rapamycin was added to purified mTORC1 and incubated at room temperature for 1 hr. As a control, 100 nM Torin-1 was added to purified mTORC1 and monitored over the same period of time. At specified time points, an aliquot of the reaction was removed and used to prepare negatively stained specimens as detailed above. Images were recorded at a nominal magnification of 52,000x on a 1K x 1K charge-coupled device (CCD) camera (Gatan) with a Philips CM10 electron microscope operated at 100 kV.

Cryo-negative staining and EM

Cryo-negative staining was performed as described (Ohi et al., 2004). In brief, glycerol was added to purified mTORC1 to a final concentration of 5%. Samples were embedded in a layer of uranyl formate between two carbon films on Quantifoil R2/1 400 mesh grids (Quantifoil Micro Tools GmbH) and frozen in liquid nitrogen. Specimens were examined using an Oxford cryo-transfer holder and a Tecnai F20 electron microscope equipped with a field emission electron source (FEI) operated at 200 kV. The same specimen areas were recorded at tilt angles of 50° and 0° on Kodak SO-163 films under low-dose conditions at a nominal magnification of 50,000 and a defocus value of $-2.5\ \mu\text{m}$. The negatives were developed for 12 min at 20°C using full-strength Kodak D-19 developer. Micrographs were digitized with a Zeiss SCAI scanner using a step size of 7 μm . 3 x 3 pixels were averaged to yield a pixel size of 4.2 Å on the specimen level. A total of 8,679 particle pairs were interactively selected from both the untilted and 50° tilted images (28 pairs) using WEB, the display program associated with the SPIDER software package. The selected particles were windowed into 128 x 128-pixel images, and the particles from the untilted specimens were classified into 10 classes as described above. Particles from three of the classes were combined (2,409 particles) and a 3D reconstruction was calculated using the backprojection, backprojection refinement and angular refinement procedures in SPIDER. The resolution of the final 3D reconstruction was estimated to be 28 Å using the Fourier Shell Correlation (FSC) = 0.5 criterion (Figure S2).

Cryo-EM and 3D reconstruction

For vitrification, Quantifoil R1.2/1.3 400 mesh grids were overlaid with a thin layer of carbon film and glow discharged. 3 μl of mTORC1 (~0.02 mg/ml) was adsorbed to a grid, and the grid was blotted and frozen in liquid ethane using a Vitrobot (FEI). Specimens were examined using a Gatan 626 cryo-holder on a Tecnai F20 electron microscope equipped with a field emission electron source (FEI) operated at 200 kV. Images were recorded untilted or at a tilt angle of 45° on Kodak SO-163 films under low-dose conditions at a nominal magnification of 50,000x and defocus values ranging from -3.5 to $-6.0\ \mu\text{m}$. The carbon film induced mTORC1 to adsorb to the grid in a preferred orientation, making it necessary to collect images of tilted specimens to obtain the

multiple views needed for 3D reconstruction. Films were developed and scanned as described above. Because of serious drifting and charging, only a very small percentage of the images recorded from tilted specimens could be used. A total of 30,494 particles were manually selected from 323 images (260 untilted, 63 tilted) using the program Ximdisp associated with the MRC program suite (Crowther et al., 1996; Smith, 1999). Particles were windowed into 100 x 100-pixel images, rotationally and translationally aligned, subjected to 10 cycles of multi-reference alignment, followed by K-means classification specifying 200 output classes. Images corresponding to the major contaminant CCT chaperonins were discarded, reducing the total number of particles to 28,325. The defocus value for each particle was deduced from its position in the image and the tilt angle, which was determined with the program CTFTILT (Mindell and Grigorieff, 2003). Using the cryo-negative stain reconstruction as initial model, individual particle images were input into FREALIGN (Grigorieff, 2007) to determine and refine the orientation parameters, to correct for the CTF, and to calculate 3D reconstructions. The resolution of the final 3D reconstruction from FREALIGN was estimated to be 26 Å using the FSC = 0.5 criterion. The density map was low-pass filtered to this resolution for display and analysis. The contouring threshold was determined from the estimated molecular mass of mTORC1 (~1.1 MDa) assuming a protein density of 0.8 Da Å⁻³. The 3D map has been deposited in the EM database at the European Molecular Biology Laboratory's European Bioinformatics Institute (accession code EMD-5197).

Raptor reconstruction

Images of negatively stained raptor were collected on a Tecnai T12 electron microscope (FEI) operated at 120 kV. Image pairs were recorded for the same specimen areas at tilt angles of 60° and 0° on imaging plates under low-dose conditions at a nominal magnification of 67,000x and a defocus value of -1.5 µm. A total of 12,216 particle pairs were interactively selected from both the untilted and 60° tilted images (83 pairs) using WEB. The selected particles were windowed into 60 x 60-pixel images, and the particles from the untilted specimens were classified into 50 classes as described above. An initial 3D reconstruction was calculated from one of the 50 classes. Particles from 2 additional

classes showing similar projection structures were added and further refinement was carried out using the angular refinement procedure in SPIDER. The resulting map was used as an input model for the program FREALIGN, which was used to further refine the orientation parameters of the individual particles and to correct for the contrast transfer function (CTF) of each particle image according to its defocus value. The defocus value for each particle was deduced from its position in the image and the tilt angle, which was determined with the program CTFTILT. The resolution of the final 3D reconstruction from FREALIGN was estimated to be 28 Å using the FSC = 0.5 criterion (Figure S3). The 3D map has been deposited to the EM database at the European Molecular Biology Laboratory's European Bioinformatics Institute (accession code EMD-5198).

Molecular docking and visualization

The raptor 3D reconstruction was manually fitted into the mTORC1 cryo-EM map using the UCSF Chimera package. mLST8 is a ~36-kDa protein that is predicted to adopt a 7-blade WD40-repeat domain structure. We used the crystal structure of WDR5 (Song and Kingston, 2008) (24% sequence identity to mLST8, PDB code: 3EMH) as a model to dock into the mTORC1 density map. All molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081) (Pettersen et al., 2004).

Supplemental References

- Crowther, R.A., Henderson, R., and Smith, J.M. (1996). MRC image processing programs. *J Struct Biol* *116*, 9-16.
- Frank, J., Radermacher, M., Penczek, P., Zhu, J., Li, Y., Ladjadj, M., and Leith, A. (1996). SPIDER and WEB: processing and visualization of images in 3D electron microscopy and related fields. *J Struct Biol* *116*, 190-199.
- Grigorieff, N. (2007). FREALIGN: high-resolution refinement of single particle structures. *J Struct Biol* *157*, 117-125.
- Ludtke, S.J., Baldwin, P.R., and Chiu, W. (1999). EMAN: semiautomated software for high-resolution single-particle reconstructions. *J Struct Biol* *128*, 82-97.
- Mindell, J.A., and Grigorieff, N. (2003). Accurate determination of local defocus and specimen tilt in electron microscopy. *J Struct Biol* *142*, 334-347.
- Ohi, M., Li, Y., Cheng, Y., and Walz, T. (2004). Negative Staining and Image Classification - Powerful Tools in Modern Electron Microscopy. *Biol Proced Online* *6*, 23-34.
- Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., and Ferrin, T.E. (2004). UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem* *25*, 1605-1612.
- Sancak, Y., Thoreen, C.C., Peterson, T.R., Lindquist, R.A., Kang, S.A., Spooner, E., Carr, S.A., and Sabatini, D.M. (2007). PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. *Mol Cell* *25*, 903-915.
- Smith, J.M. (1999). Ximdisp--A visualization tool to aid structure determination from electron microscope images. *J Struct Biol* *125*, 223-228.
- Song, J.J., and Kingston, R.E. (2008). WDR5 interacts with mixed lineage leukemia (MLL) protein via the histone H3-binding pocket. *J Biol Chem* *283*, 35258-35264.
- 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 mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. *J Biol Chem* *284*, 8023-8032.