Mechanisms of mTORC1 activation by RHEB and inhibition by PRAS40

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The mechanistic target of rapamycin complex 1 (mTORC1) controls cell growth and metabolism in response to nutrients, energy levels, and growth factors. It contains the atypical kinase mTOR and the RAPTOR subunit that binds to the Tor signalling sequence (TOS) motif of substrates and regulators. mTORC1 is activated by the small GTPase RHEB (Ras homologue enriched in brain) and inhibited by PRAS40. Here we present the 3.0 ångström cryo–electron microscopy structure of mTORC1 and the 3.4 ångström structure of activated RHEB–mTORC1. RHEB binds to mTOR distally from the kinase active site, yet causes a global conformational change that allosterically realigns active-site residues, accelerating catalysis. Cancer–associated hypermutating activations map to structural elements that maintain the inactive state, and we provide biochemical evidence that they mimic RHEB relieving auto–inhibition. We also present crystal structures of RAPTOR–TOS motif complexes that define the determinants of TOS recognition, of an mTOR FKBP12–rapamycin–binding (FRB) domain–substrate complex that establishes a second substrate–recruitment mechanism, and of a truncated mTOR–PRAS40 complex that reveals PRAS40 inhibits both substrate–recruitment sites. These findings help explain how mTORC1 selects its substrates, how its kinase activity is controlled, and how it is activated by cancer–associated mutations.

mTORC1 controls multiple aspects of cell growth and homeostasis, including protein synthesis, lipogenesis, glucose metabolism, autophagy, lysosome biogenesis, proliferation, and survival, in response to environmental cues ranging from levels of amino acids, glucose, energy, and oxygen to growth factors1–3. mTORC1 exhibits multiple levels of subcellular localization and regulation4,5. mTOR is a serine–threonine kinase required for the treatment of cancer4,5. mTORC1 is an approximately 1-MDa homologue enriched in brain) and inhibited by PRAS40. Here we present the 3.0 ångström cryo–electron microscopy structure of mTORC1 and the 3.4 ångström structure of activated RHEB–mTORC1. RHEB binds to mTOR distally from the kinase active site, yet causes a global conformational change that allosterically realigns active-site residues, accelerating catalysis. Cancer–associated hypermutating activations map to structural elements that maintain the inactive state, and we provide biochemical evidence that they mimic RHEB relieving auto–inhibition. We also present crystal structures of RAPTOR–TOS motif complexes that define the determinants of TOS recognition, of an mTOR FKBP12–rapamycin–binding (FRB) domain–substrate complex that establishes a second substrate–recruitment mechanism, and of a truncated mTOR–PRAS40 complex that reveals PRAS40 inhibits both substrate–recruitment sites. These findings help explain how mTORC1 selects its substrates, how its kinase activity is controlled, and how it is activated by cancer–associated mutations.

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We therefore mutated one or more hydrophobic residues in each substrate in the context of 20-residue synthetic peptides. The mutations reduced phosphorylation of all four substrates by a factor of 4–13, consistent with these substrates using the FRB docking site to enter the catalytic cleft (Fig. 1g).

We also investigated 4EBP1, whose Thr37 and Thr46 phosphorylation sites are followed by an amphipathic helix that binds to eIF4E\(^{[10]}\). Appreciable Thr37 phosphorylation required extending the peptide to the first turn of the amphipathic helix, which when mutated reduced phosphorylation back to a barely detectable level (Fig. 1g). With the Thr46 site, the 20-residue peptide that reaches partway to the amphipathic helix was phosphorylated approximately fivefold less than a peptide encompassing the entire helix. In this longer peptide, mutation of the first and second sets of hydrophobic residues reduced phosphorylation by a factor of approximately 8 and 3, respectively (Fig. 1g). Consistent with the amphipathic helix being recruited by the FRB, addition of eIF4E, which sequesters the helix, reduced full-length 4EBP1 phosphorylation by both mTORC1 and mTOR\(^{\Delta N}\)–mLST8 by a factor of approximately 4 (Fig. 1h and Extended Data Fig. 1d).

**Crystal structures of Raptor–TOS motif complexes**

We determined the crystal structures of Arabidopsis thaliana Raptor (atRaptor) bound to TOS motif peptides from human 4EBP1, S6K1, and PRAS40, at 3.0, 3.1, and 3.35 Å resolution, respectively, and apo–atRaptor at 3.0 Å (Fig. 2a and Extended Data Table 1). The atRaptor residues that contact the TOS peptides are identical in human RAPTOR, and the atRaptor–4EBP1 TOS interface (Fig. 2b) does not differ discernibly in the 3.0 Å human mTORC1–4EBP1 cryo-EM structure described below (Extended Data Fig. 2).

RAPTOR has a sausage-like shape, with the N-terminal caspase-homology domain\(^{[4]}\) at one end, an \(\alpha\)–\(\alpha\) solenoid of approximately eight armadillo repeats in the middle, and a C-terminal WD40 domain at the other end (Fig. 2a). The caspase-homology domain can be superimposed on caspase6 with a root-mean square deviation of approximately 3 Å for 175 C\(_\alpha\) atoms, although RAPTOR lacks the caspase Cys–His catalytic dyad\(^{[5]}\) (Supplementary Discussion). The TOS peptides bind to a groove between the caspase fold and the solenoid, approximately 65 Å away from the kinase active site (Fig. 1a). One side of this groove is formed by a four-helix insertion in the caspase fold, and the other side by the first three armadillo repeats (Fig. 2a).

All three TOS peptides have an equivalent eight-residue segment ordered in the crystals (Extended Data Fig. 3a–d). The key phenylalanine side chain of the TOS consensus\(^{[11,12]}\) FX\(\Phi\)DX\(\Phi\) (\(\Phi\) hydrophobic, X any residue) binds to a pocket together with the preceding TOS residue (hereafter F\(_1\) position). In 4EBP1, this unit consists of the Phe114 side chain, whereas in S6K1 and PRAS40 the Phe5 and Phe129 aromatic rings make functionally analogous van der Waals contacts with Van4 and Leu128, respectively (Fig. 2b–d). In addition, the phenylalanine backbone amide group hydrogen bonds to Tyr475 (human RAPTOR numbering). Alanine mutation of the 4EBP1 Glu113 reduces its affinity for human RAPTOR by a factor of 20, confirming the importance of its stacking with the phenylalanine and recognition of the residue pair as a unit (dissociation constant (\(K_D\)) values in Extended Data Fig. 3e, f).

The other conserved TOS residues make overall conserved RAPTOR contacts (Fig. 2b–d). The hydrophobic F\(_3\) side chain (Met116, Ile7, and Met131 of 4EBP1, S6K1, and PRAS40, respectively) binds into a tight pocket at the bottom of the groove, and accordingly its alanine mutation reduces binding by nearly two orders of magnitude (Extended Data Fig. 3e). The F\(_3\) aspartic acid side chain forms a hydrogen bond network involving Arg305 and the F\(_4\) backbone carbonyl group, and its mutation increases the \(K_D\) fivefold (Extended Data Fig. 3d, e). By contrast, the hydrophobic F\(_4\) side chain (4EBP1 Ile118 and S6K1 Leu9; PRAS40 Glu133 is disordered) binds to a shallow, solvent-exposed surface pocket, and its mutation reduces binding only modestly (Extended Data Fig. 3e).

Consistent with the conservation of contacts in the three structures, the PRAS40 inhibitor has a TOS–RAPTOR \(K_D\) similar to those of S6K1.
As expected, deletion of TOS (PRAS40\textsubscript{173–256}) had no effect, but deletion revealed two PRAS40 anchor points separated by an unstructured to a continuum of conformational states. One class with approximately 20% of the particles had an overall conformation distinct from the ensemble of the other classes, and this was the only class that contained RHEB, one on each mTOR of dimeric mTORC1 (Extended Data Fig. 5c–e).

Because of the conformational flexibility between and within the two mTOR–RAPTOR–mLST8 complexes (Extended Data Fig. 5c), we converted the particles to monomers with partial signal subtraction\textsuperscript{34}, and calculated focused reconstructions with three partly overlapping masks (2.98, 2.95, and 2.96 Å; Extended Data Fig. 5a, b). Using these three

- PRAS40 blocks the FRB substrate–recruitment site

To identify additional mTORC1-binding elements of PRAS40, we first tested whether the PRAS40 segment reported to be necessary for inhibition\textsuperscript{14} (PRAS40\textsubscript{114–256}) can also inhibit mTOR\textsuperscript{ΔN–mLST8–phosphorylation} S6K1\textsubscript{167–404}. PRAS40\textsubscript{114–256} inhibited this TOS-independent reaction with an apparent inhibitor constant (K\textsubscript{i}) of approximately 52 μM (calculated from half-maximum inhibitory concentration (IC\textsubscript{50}) values; Fig. 3a), which is significantly lower than the approximately 430 μM K\textsubscript{i} of S6K1\textsubscript{167–404} (Extended Data Fig. 1b).

As expected, deletion of TOS (PRAS40\textsubscript{173–256}) had no effect, but deletion of 33 additional residues (PRAS40\textsubscript{206–256}) reduced inhibition by a factor of approximately 4 (Fig. 3a). Very similar results were obtained with the 4EBP1\textsubscript{12–24} substrate (Extended Data Fig. 4a).

We next determined the 3.4 Å co-crystal structure of PRAS40\textsubscript{173–256} bound to mTOR\textsuperscript{ΔN–mLST8} (Extended Data Fig. 4f). The structure revealed two PRAS40 anchor points separated by an unstructured segment: an amphipathic α-helix (residues 212–232) bound to the FRB domain and a β-strand (residues 188–196) bound to the mLST8 WD40 domain (Fig. 1a and Extended Data Fig. 4b).

The amphipathic helix binds to the same FRB site as the S6K1 substrate, but at five turns it is substantially longer and makes more extensive contacts than S6K1 (Fig. 3b, c and Extended Data Fig. 4c). PRAS40 uses the Met222 side chain to bind to the same rapamycin-binding pocket as the S6K1 Leu396 (Fig. 3c), and five additional hydrophobic side chains (Leu215, Ile218, Ala219, Leu225, and Val226) to contact an extended FRB surface.

The PRAS40 β-strand has a phenylalanine side chain (Phe193) inserting into a pocket between two mLST8 β-propeller blades (Tyr195, Trp197, Pro167, Pro212), and its peptide backbone makes three β-sheet hydrogen bonds to the edge of one β-propeller (Fig. 3d and Extended Data Fig. 4d). These PRAS40 interactions are consistent with reduced inhibition by PRAS40\textsubscript{206–256}, which lacks the β-strand, compared with PRAS40\textsubscript{173–256} (Fig. 3a).

We further confirmed the importance of the amphipathic helix by mutating Met222 and four additional FRB-interacting residues (L215A, I218A, A219G, L225A) in full-length PRAS40. As shown in Fig. 3e, the mutations reduced inhibition of mTORC1 phosphorylating full-length 4EBP1 by a factor of approximately 50.

- mTORC1 and RHEB–mTORC1 cryo–EM structures

To address how RHEB activates mTORC1, we collected cryo-EM data on mTORC1 that was crosslinked in the presence of excess RHEB–GTP–S and 4EBP1 (Extended Data Fig. 2c). The 3D auto-refinement of 580,768 particles in C\textsubscript{2} symmetry led to a consensus reconstruction extending to 3.2 Å resolution, as determined by the gold-standard Fourier shell correlation (FSC) procedure\textsuperscript{32} (Extended Data Fig. 5a, b).

In subsequent 3D classification in C\textsubscript{2}, most classes appeared to belong to a continuum of conformational states. One class with approximately
reconstructions with the composite map option of REFMAC5 (ref. 34), we refined mTORC1 at 3.0 Å resolution (Extended Data Figs 2c, 5f, 6 and 7a). Using the same procedure, we refined RHEB–mTORC1 at 3.4 Å (Extended Data Figs 2c and 5a, b).

As with the mTORΔN–mLST8 crystal structure23, the PIKK-specific FAT domain adopts a C-shaped solenoid structure that clamps onto the kinase domain, with the start of the solenoid interacting with the kinase domain N-lobe, and its end with both the N- and C-lobes (Fig. 4a). In keeping with the secondary structures of previous mTOR cryo-EM reconstructions24,35, the N-terminal segment missing from mTORΔN23 starts with an α–α solenoid of 18 HEAT repeats (N-heat), followed by a smaller middle solenoid of 7.5 HEAT repeats (M-heat), and an approximately 110 residue helical-repeat segment that is structurally contiguous with the subsequent FAT domain, whose middle portion is anchored on it. The intra-FAT conformational change is entirely by an approximately 30-fold increase in the catalytic constant kcat (Asp2338 and His2340) (Fig. 5e, f and Extended Data Fig. 8b, c). The FAT residues 1261–1266 exhibit smaller rotations (Fig. 5c). These conformational changes in the FAT are coupled to its C-terminal portion moving away from the kinase, the N-lobe of the kinase moving in to the space vacated by the FAT, the FAT–N-lobe interface repacking into a looser arrangement, and the catalytic cleft between the N- and C-lobes closing by 8° (Fig. 5c, d and Supplementary Video 2).

**RHEB allosterically activates mTORC1**

In apo-mTORC1, the N-heat RHEB-binding site is far away from those on M-heat and FAT, displaced by approximately 18 Å relative to its position in the RHEB-bound state (Fig. 5a). On RHEB binding, the N-heat solenoid swings towards M-heat through an approximately 19° rotation, reconstituting the RHEB-binding site and inducing new interactions between the N-terminal portions of N-heat and FAT (Figs 4c and 5a and Supplementary Video 1). This causes a conformational change within the FAT domain, whose middle portion gets twisted and dragged by the moving N-heat solenoid end that is anchored on it. The intra-FAT conformational change is entirely distinct from the conformational flexibility apo-mTORC1 exhibits (Extended Data Fig. 7f and Supplementary Discussion). The two conformations are incompatible in a mixed dimer, as this would require a greater than 20 Å offset in the N-heat portion of the dimerization interface. This explains the lack of single-RHEB 3D classes, and suggests that two RHEB molecules bind to mTORC1 cooperatively. In support of this, we find that the RHEB–GTP–S response curve of mTORC1 phosphorylating 4EBP1 best fits a Hill slope model with a Hill coefficient of approximately 2.0 (Fig. 5b; the approximately 100 µM half-maximum effective concentration (EC50) in solution is probably not reflective of the membrane-surface reaction in vivo23).

The intra-FAT conformational change occurs at hinge regions that allow for relative rotations of flanking segments. One hinge around residue 1443 is associated with a major rotation of 30° between the FAT sub-domains TRD1 and TRD2 (ref. 23), and two other hinges exhibit smaller rotations (Fig. 5c). These conformational changes in the FAT are coupled to its C-terminal portion moving away from the kinase, the N-lobe of the kinase moving in to the space vacated by the FAT, the FAT–N-lobe interface repacking into a looser arrangement, and the catalytic cleft between the N- and C-lobes closing by 8° (Fig. 5c, d and Supplementary Video 2).

The closing of the catalytic cleft changes the relative orientation of the ATP-contacting and catalytic residues from the N- and C-lobes. This brings the ATP phosphate groups that are bound by the N-lobe into closer proximity with critical C-lobe residues that include the Mg2+ ligands23 (Asn2343 and Asp2357) and the two catalytic residues23 (Asp2338 and His2340) (Fig. 5e, f and Extended Data Fig. 8b, c).

This indicates that RHEB activates mTORC1 by allosterically realigning active-site residues, bringing them into the correct register for catalysis. To confirm this, we compared the steady-state kinetic constants of S6K167–69 phosphorylation by mTORC1 in the presence of 250 µM RHEB–GTP–S or RHEB–GDP. Activation is accounted for entirely by an approximately 30-fold increase in the catalytic constant kcat, from 0.09 s−1 to 2.9 s−1, whereas Km values remain essentially unchanged (Fig. 5g). RHEB similarly increased the apparent kcat of full-length 4EBP1 phosphorylation, including under single-turnover conditions, the latter indicating that the kcat effect involves the catalytic step and not a hypothetically rate-limiting product-release step (Extended Data Fig. 8d, e). RHEB–GTP–S also accelerated idle ATP hydrolysis, a low-level activity common in protein kinases as well as PI3K37 (Extended Data Fig. 8f).

**Cancer–associated hyperactive mTOR mutants**

Cancer-associated hyperactivating mutations5,38–40 predominantly involve structure-stabilizing residues. They cluster at the major intra-FAT hinge, the FAT–N-lobe packing transition, and the N-lobe anchor in a pocket between the C-lobe and FAT, suggesting that they act by...
lowering the barriers to the N-lobe adopting the active conformation, mimicking the effects of RHEB (Fig. 6a and Extended Data Fig. 9a–c).

This hypothesis predicts that the mutations should lower the EC50 of activation by RHEB, as part of the RHEB–mTORC1 binding energy must be used to affect the conformational change, and that they should not synergize with saturating RHEB. To test these predictions, we transiently expressed and purified four representative hyperactive mTORC1 mutants5,38. A1459P in the middle of a helix at the major intra-FAT hinge, T1977R buried at the FAT–N-lobe transition, and S2215Y and E2419K at the N-lobe–C-lobe and juxtaposed C-lobe–FAT interfaces, respectively (Fig. 6a and Extended Data Fig. 9a–c).

After confirming that the mutations increase the $k_{cat}$ of mTORC1 phosphorylating S6K1367–404 without affecting the $K_m$ (Extended Data Fig. 9d), we assessed their RHEB–GTP–S dose–response curves. All four mutations shifted the response curve to lower RHEB concentrations compared with wild-type mTORC1, produced as the mutants (Fig. 6b). A1459P, S2215Y, and E2419K reduced the EC50 comparably, by factors of 6.6, 7.0, and 7.4, respectively, whereas T1977R reduced it by a factor of 4.0 (Fig. 6b). Importantly, the mutations did not synergize with RHEB, as at the highest, nearly saturating RHEB concentration, the mutants exhibited S6K1367–404 phosphorylation levels within approximately 15% of the wild-type control (reaction velocity/enzyme concentration, $v/[E]$, of 3.7 s$^{-1}$ for wild-type, and 4.1–4.4 s$^{-1}$ for the mutants; Fig. 6b).

Conclusion

We show that the TOS motif docking site is approximately 65 Å from the kinase active site, suggesting that it acts to increase the effective substrate concentration, and we establish a second substrate-docking site corresponding to the rapamycin-binding site at the entrance of the catalytic cleft. PRAS40 binds to both substrate-docking sites and an additional site on mLST8 to achieve inhibition. We also show that the low kinase activity of apo-mTORC1 is due to a misalignment of the kinase N- and C-lobes and their associated ATP-binding and catalytic residues. The FAT clamp, present in all PIKKs, is a key auto-inhibitory element that keeps the N-lobe misaligned. RHEB, binding approximately 60 Å away from the active site, induces a movement of the N-lobe domain, which pulls and twists the FAT clamp, freeing the N-lobe to adopt the active conformation. The end result of this process is probably mimicked by cancer-associated mutations that activate mTOR.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Protein expression and purification. For the FRB–S6K fusion protein, a synthetic gene encoding the FRB domain of human mTOR (residues 2018–2114) followed by a three-amino-acid linker (SGG) and residues 389–414 of human S6K1–Δ was cloned into a modified pGEX4T3 vector. The fusion protein was overexpressed in the Escherichia coli strain BL21 (DE3), and was purified by glutathione-affinity chromatography, removal of the GST tag with TEV protease, and fractionation by ion exchange and size-exclusion chromatography. The peak fractions were concentrated to 40 mg ml−1 in 20 mM Tris·HCl, pH 8.0, 0.5 mM NaCl, 10% glycerol and 0.5 mM tris (2-carboxyethyl) phosphine (TCEP).

Human S6K1 was overexpressed in insect cells with a baculovirus expressing the GST-tagged protein and purified similarly as described previously. The corresponding mTOR plasmids were cloned into the pcDNA3.1(+) vector. The plasmids were transfected into HEK293F cells as described for the initial PRAS40–mTOR–mLST8 and SeMet–PRAS40-containing complex. 30 μM mTOR was incubated with 0.2 μM of truncated mTORC1, followed by addition of 40 mM MgCl2. Both states of charged RHEB were then crosslinked using both isomorphous and dispersive differences. The atRaptor used in crystallization experiments were purchased from Peptide 2.0.

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Human S6K1 (residues 883–942) was produced by infecting High Five insect cells with a strain BL21(DE3), and was purified by glutathione-affinity chromatography, removal of the GST tag with TEV protease, and fractionation by ion exchange and size-exclusion chromatography. The peak fractions were concentrated to 40 mg ml−1 (≈2 μM) and stored in small aliquots in −80 °C.

To prepare nucleotide-bound RHEB, RHEB was overexpressed and purified similarly as FRB–S6K fusion protein. To charge RHEB with GDP, the protein was first dialysed overnight against a buffer containing 20 mM Tris·HCl, pH 8.0, 300 mM NaCl, 5% glycerol, 5 mM EDTA, 0.5 mM TCEP, and 100 mg ml−1 acridine-activated charcoal (Sigma). Next, the protein was incubated with 30-fold molar excess of GDP (Sigma–Alrich) on ice for 30 min following addition of 15 mM MgCl2. To charge RHEB with GTP·S, the protein was incubated with 30-fold molar excess of GTP·S (Sigma, 20 mM EDTA, and 10 units of alkaline phosphatase (New England BioLabs) per milligram of RHEB at 37 °C for 1 h followed by addition of 40 mM MgCl2. Both states of charged RHEB were then purified by size-exclusion chromatography (Superdex 75) in 20 mM bicine, pH 8.0, 200 mM NaCl, 5% glycerol, 5 mM MgCl2, and 0.1 mM TCEP. The peak fractions were concentrated to 25–40 mg ml−1.

Cryocrypsis and mTORC1 complex crystals were grown by the hanging-drop vapour diffusion method at 16 °C from 100 mM bis-tris propane, pH 7.0, 30% Tacsimate (Hampton Research). Crystals were transferred to 100 mM bis-tris propane, pH 7.0, 6.3 M NaFormate and flash-frozen in liquid nitrogen.

The atRaptor native crystals were grown at 4 °C by the hanging-drop vapour diffusion method from a crystallization buffer of 100 mM HEPES, pH 7.5, 50 mM Ca(OAc)2, 4–8% PEG 8000, 0.5 mM TCEP. For heavy-atom derivatives, crystals were soaked in 0.4–0.8 M thimerosal for 2 h before cryo-protection (not shown).

For the initial PRAS40–mTOR–mLST8 and SeMet–PRAS40-containing complex, 30 μM mTOR was incubated with 0.2 μM of truncated mTORC1, followed by addition of 40 mM MgCl2. Both states of charged RHEB were then crosslinked using both isomorphous and dispersive differences. The atRaptor used in crystallization experiments were purchased from Peptide 2.0.

Crystal structure determination and refinement. For the FRB–S6K1 complex, initial phases were obtained by molecular replacement with PHASER using the FRB structure (Protein Data Bank accession number 1FA9) as the search model. The FRB–S6K1 and other crystal structures described in this study were built using O19 and refined with REFMAC5 (ref. 42) and PHENIX44. The FRB–S6K1 model has a Molprobity clashscore of 0.36. The Ramachandran plot has 99.8, 0.2, and 0% of the residues in the favoured, allowed, and outlier regions, respectively.

For atRaptor, initial phases were calculated from two thimerosal derivatives using both isomorphous and dispersive differences. The atRaptor used in crystallization experiments were purchased from Peptide 2.0.

Cryo-EM sample preparation and data collection. Cryo-EM samples were prepared using two different cryocooling procedures. In the initial, high-salt cryocooling procedure, 0.42 μM mTORC1 was incubated with 250 μM RHEB–GTP·S, 10 μM 4EBP1, 0.2 mM GTP·S, 1 mM AMP–PNP for 10 min, and crosslinked with 0.18 (v/v) glutaraldehyde for 45 min on ice in 20 mM bicine, pH 8.0, 300 mM NaCl, 10% glycerol, 5 mM MgCl2, and 0.5 mM TCEP. The reaction was quenched with 100 mM Tris·HCl, pH 8.0, and the mixture was purified by size-exclusion chromatography (Superose 6) in 20 mM Tris·HCl, pH 8.0, 260 mM NaCl, 5 mM MgCl2, and 0.5 mM TCEP. Peak fractions were concentrated by ultrafiltration to 1 mg ml−1 and were supplemented with 100 μM RHEB–GTP·S, 10 μM 4EBP1, 0.2 mM GTP·S, and 1 mM AMP–PNP. The sample (3 μl) was applied to glow-discharged UltrAuFoil 300 mesh R1.2/1.3 grids (Quantifoil). Grids were blotted for 2.5 s at 22 °C and approximately 95% humidity and plunge-frozen in liquid ethane using an FEI Vitrobot Mark IV.
After determining that high ionic strength severely reduced RHEB activation (not shown), we reduced the salt concentration and modified the crosslinking procedure by first reducing the concentrations of mTORC1, RHEB–GTP–S, and NaCl to 0.21 μM, 120 μM, and 100 mM, respectively, then crosslinking with 0.24 mM BS3 for 45 min on ice, followed by the addition of 130 μM RHEB and 160 mM NaCl and further crosslinking with 0.18% glutaraldehyde for 45 min. Quenching and purification were performed as with the high-salt procedure in 260 mM NaCl. The main dataset used for apo-mTORC1 reconstruction was collected on the New York Structural Biology Center Simons Electron Microscopy Center using LIGER 45 on a Titan Krios microscope operated at 300 kV and equipped with a Gatan K2 Summit camera using defocus values ranging from −1.2 to −3 μm. The camera was operated in counting mode with a 1.331 Å pixel size at the specimen level and a dose rate of 8.3 electrons per pixel per second. Each 12×-s exposure was dose-fractionated into 60 frames and contained a total dose of approximately 56 electrons per square angstrom. This dataset, which was acquired over 7 sessions, consisted of 4,502 micrographs from high-salt crosslinked samples and 3,740 from lower-salt crosslinked samples. The second dataset, which used only the lower-salt crosslinking condition, was collected at the Sloan Kettering Institute Titans microscope/Gatan K2 Summit camera operated at 300 kV with a 1.089 Å pixel size and 8.0 electrons per pixel per second. Each 8×-s exposure was dose-fractionated into 40 frames and contained a total dose of approximately 52 electrons per square angstrom. This dataset consisted of 8,354 micrographs.

**Cryo-EM image processing.** Motion correction was performed with MOTIONCORR and MOTIONCORR-2 (ref. 46) for the first and second datasets, respectively. Contrast transfer function parameters were estimated with the CTFEFD4 (ref. 47), and subsequent 2D/3D classifications and 3D refinements were performed with RELION-1.4 (ref. 48) and RELION-2.0 (ref. 49). All reported resolutions are from gold-standard refinement procedures with the FSC = 0.143 after post-processing by applying a soft mask, correction for the modulation transfer function of the detector, temperature-factor sharpening, and correction for FSC curves to account for the effects of the soft mask as implemented in RELIEX 48,49. Initial references for template-based particle picking were from 2D-class averages of manually picked particles. Multiple rounds of 2D and, for some data subsets, 3D classifications were then used to remove false positives and particles from classes with poor contrast. In the high-salt dataset, a total of 580,768 particles were retained (Extended Data Fig. 5a). After an initial 3D auto-refinement with C2 symmetry, the particles were improved by particle-based motion correction and radiation-damage weighting. The resulting ‘polished’ particles were used for the 3D auto-refinement of a consensus mTORC1 dimer map in C2, yielding a 3.23 Å reconstruction (Extended Data Fig. 5a). After 3D classification in point group C2, 114,879 (19.7%) of the polished particles clustered to a single RHEB–containing class. The fraction of RHEB-containing particles was approximately 2.5-fold higher in the low-salt crosslinked particles than the high-salt ones. The remaining six apo-mTORC1 3D classes appeared to sample a continuum of conformational flexibility, both between and within the two mTORC1–RAPTOR–mLST8 complexes (Extended Data Fig. 5a). Because of this conformational flexibility, we converted the particles to a ‘monomeric’ form (illustrated in Extended Data Fig. 5a). For this, we duplicated the particle list and advanced the RELION rot angle by 180° to extract the signal of the second copy of the complex superimposed on the first. We then subtracted the signal from the masked map of the second mTORC1–RAPTOR–mLST8 complex in the consensus reconstruction (before post-processing) from each particle in the combined set as described 45. Three-dimensional auto-refinement then yielded a monomeric mTORC1 consensus reconstruction to 3.11 Å. An alternative, ‘pseudo-monomer’ set of particles was calculated by switching the two N-heat domains, as this domain seemed to modulate mTORC1 reconstructions assigned to the following coordinates: N-heat, and for RHEB–mTORC1, RHEB–GTP–S, and minimal RHEB–interacting M-heat and FAT elements of protomer 2. The dimeric mTORC1–RHEB reconstruction was to 3.8 Å, the monomers to 3.58 Å, and the three focused 3D refinements to 3.4, 3.41, and 3.38 Å (Extended Data Fig. 5a). The inclusion of the down-scaled particles from the second dataset improved the resolution limits only marginally, but the maps had better continuity, especially in the relatively less ordered regions.

**Cryo-EM structure refinement.** Model refinement was done with REFMAC5 modified for cryo-EM 44, with a composite map of the three focused reconstructions assigned to the following coordinates: N-heat, and for RHEB–mTORC1, RHEB–GTP–S, and minimal RHEB–interacting M-heat and FAT elements of protomer 2 (both duplicated) to the third focused map; M-heat (961–1222), RAPTOR, TOS, and RAPTOR-bound β-strand from M-heat–FAT linker to the second focused map; M-heat (933–960), FAT–kinase domain (1261–2549), AMPNP, mLST8, and for RHEB–mTORC1, RHEB–GTP–S, and minimal RHEB–interacting regions of N-heat (duplicated) to protomer 2 to the third focused map. The three focused maps were aligned on the corresponding regions of the consensus C2 map by first obtaining the rotation–translation matrix with CHIMERA 43 and then applying the transformation with CCP4 (ref. 42). The resulting composite maps were used for building a model of the monomeric complex using O 41, and for refinement with REMAC5 (ref. 42) and PHENIX 44. The apo-mTORC1 monomer was refined to 3.0 Å with weak secondary structure restraints generated by ProSMART 32, and the RHEB–mTORC1 monomer to 3.4 Å with tighter secondary structure restraints. RHEB was built on the basis of the published structure 46. Validation refinement was done as described 44. To refine the dimeric complexes, two copies of the three focused maps of each complex were aligned on the corresponding C2 maps as above. The six resulting maps for each complex were then combined with the consensus reconstruction to construct the high-resolution structures described as described for the monomers. The dimeric apo-mTORC1 and RHEB–mTORC1 models were refined against these structure factors using tight non-crystallographic symmetry restraints for the positions and B-factors of the atoms.

**In vitro kinase assays.** In vitro kinase assays were performed as described 44, except reaction duration was 20 min. In brief, reactions were assembled in a buffer of 25 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM MgCl2, 2 mM dithiothreitol, 5% (v/v) glycerol and allowed to incubate on ice for 5 min. Reactions were started by the addition of cold ATP (0.5 mM final concentration except for Extended Data Fig. 5f, which were added at the indicated concentrations) supplemented with 2–4 μCi [γ-32P]ATP (100 μCi/μmol) 3,5-ATPase assay. Reactions with RHEB–GTP–S or RHEB–GDP were supplemented with 200 μM of the corresponding nucleotide. For reactions with short peptide substrates (Fig. 1g), 0.01% Triton X-100 (Sigma) was added to reduce non-specific interaction of peptides with the test tube. Reactions with peptide substrates were resolved on 16% or 19% tricine–urea SDS–PAGE gels. The K_i values of PRAS40 fragments inhibiting S6K1 367–404 phosphorylation shown in Fig. 3a were calculated using the IC50 values, the 10 μM S6K1 367–404 substrate concentration, and the 430 μM K_M of this substrate peptide (Extended Data Fig. 1b according to the competitive inhibitor equation K_i = IC50/(1 + ([S]/K_M)).

**In vitro S6K1 phosphorylation assay.** HA-S6K1 wild type and mutants were cloned into the pcDNA3.1(+) vector. HEK293F cells (Invitrogen) were maintained in DMEM medium with 10% fetal bovine serum (Sigma) at 37 °C and 5% CO2. For transfection, cells were seeded into six-well tissue culture plates, cultured to 70% confluence, and exchanged into fresh medium 1 h before transfection. Cells were transfected with 2 μg each of HA-S6K1 wild-type or mutant plasmids using HEK293F cells (Invitrogen) were maintained in DMEM medium with 10% fetal bovine serum (Sigma) at 37 °C and 5% CO2. For transfection, cells were seeded into six-well tissue culture plates, cultured to 70% confluence, and exchanged into fresh medium 1 h before transfection. Cells were transfected with 2 μg each of HA-S6K1 wild-type or mutant plasmids using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, cells were lysed in 50 mM Tris-HCl, 150 mM NaCl, 0.5 mM TCEP, 1% Triton-X100, 2 mM EDTA, 50 mM NaF, 10 mM β-glycerophosphate, and 10 mM Na-pyrophosphate, pH 7.5, and one tablet each of Complete protease inhibitor and PhosSTOP cock -tail, then sonicated for 10 min. Supernatants were recovered, mixed with extraction buffer and NuPAGE LDS sample loading buffer (Invitrogen), and boiled for 5 min. Twenty micrograms of whole-cell extract were loaded on gel for immunoblotting with anti-HA antibody (Santa Cruz, SC805) or anti-phospho-S6K1 (T389) antibody (Cell Signaling, 20925). The immunoblots were quantified by normalizing the anti-phospho-S6K1 signal to the anti-HA signal of each reaction.

**ATPase assay.** The ATP hydrolysis assays were set up similarly as the in vitro kinase assays, except without mTOR substrates. To vary the final ATP concentrations, cold ATP was serially diluted and supplemented with [γ-32P]ATP (4 μCi/G per reaction). The reaction was initiated by mixing the ATP with the enzyme (10 μl total volume),
incubated for 20 min at 30 °C, and stopped by adding 10 μl of 2 M formic acid. Two microlitres of each reaction were then spotted on a PEI Cellulose TLC plate (Millipore), developed in 1 M formic acid and 0.5 M LiCl, dried, and quantified by phosphorimaging.

**Fluorescence polarization.** FITC (fluorescein isothiocyanate)-labelled TOS peptides were purchased from Peptide 2.0. Peptides were quantified by absorption at 495 nm by more than 20-fold dilution in 10 mM Tris-HCl, pH 8.0, using an extinction coefficient of 75,000 cm$^{-1}$ M$^{-1}$. A series of 60-μl binding conditions using serially diluted protein with 20 nM FITC-labelled TOS peptides was set up in buffer consisting of 10 mM Tris-HCl, 100 mM NaCl, 2.5% glycerol, and 1 mM TCEP, pH 8.0. Each binding condition was set up in triplicate and equilibrated at room temperature for 15 min. The fluorescence anisotropy measurements were taken with a Cary Eclipse Fluorescence Spectrophotometer with automated polarization accessory (Agilent Technologies), using 485-nm excitation (5-nm slit) and 512-nm emission (10 nm slit) wavelengths, and a G factor of 1.5111. The apparent dissociation constants ($K_d$) were obtained by fitting the data to a one-site binding model, by minimizing the sum of the square of the differences.

**Data availability.** The cryo-EM maps, including the three focused reconstruction maps and the structure factors of their composite map used in model refinement, and the refined atomic models have been deposited in the Electron Microscopy Data Bank (EMDB) and the Protein Data Bank (PDB), under accession numbers EMDB-7087 and PDB 6BCX for apo-mTORC1, and EMDB-7086 and PDB 6BCU for RHEB–mTORC1. The coordinates and structure factors of the FRB–S6K1 complex (5WBH), atRaptor (5WBI), atRaptor–4EBP1TOS (5WBJ), atRaptor–S6K1TOS (5WBK), atRaptor–PRAS40TOS (5WBL), mTOR$^{AN}$–mLST8–PRAS40$^{173–256}$ (5WBU), and mTOR$^{AN}$–mLST8–PRAS40$^{114–207}$ (5WBY) have been deposited in the Protein Data Bank. All other data are available from the corresponding author upon reasonable request.

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Extended Data Figure 1 | Substrate recruitment by the FRB domain.
a, Deletion mapping of S6K1 FRB-binding motif polypeptides (2 μM) using phosphorylation by the mTORΔN–mLST8 (30 nM) as the assay, extending previous findings23. Truncation up to 7 residues N-terminal to the Thr389 phosphorylation site (indicated by asterisk) has minimal effect, whereas C-terminal truncations starting 15 residues from Thr389 successively reduce phosphorylation. The polypeptides were produced as described in Methods for the S6K1367–404 peptide. Column graph shows velocity divided by enzyme concentration from the quantification of the 32P autoradiogram. Columns show means and markers show values from independent experiments (n = 6 for 382–410, 367–392, 367–398, and 367–410 reactions, n = 5 for 382–402, n = 4 for 367–404, and n = 3 for 367–402). The column labelled f.l. S6K1 shows the phosphorylation level of full-length S6K1 (ki superscript indicates the kinase-inactive K100R mutant) under the same conditions, as reported in ref. 23. Truncation of the S6K1 peptide to 20 residues (S6K1 382–402), which is the standardized length used in the peptide library of Fig. 1g, reduces phosphorylation to approximately 20% of S6K1367–404, probably in part because of end-effects destabilizing the helix as well as eliminating some minor contacts.
b, Michaelis–Menten steady-state kinetic constants for mTORΔN–mLST8 (30 nM) phosphorylating wild-type and the indicated mutant S6K1367–404 peptides, quantified as in a. Graph shows means (dashes) and values (markers as indicated) from independent experiments (n = 3, except for the 1, 2, 10, 750, 900, and 1,200 μM points, which are n = 2). Also shown are the K_m and k_cat values, calculated by nonlinear regression fitting of the data, above the graph, and their simulated curves in the graph. Mutations that significantly reduce phosphorylation but do not make substantial direct FRB contacts include S394A, which eliminates a hydrogen bond that stabilizes the helix N terminus, and the helix-breaking V395G mutation, which further reduces phosphorylation compared with V395A (Fig. 1d); together, these point to the importance of the helical conformation. Additional mutations include Val391 and Pro393, in the segment between Thr389 and the start of the helix. Pro393 may be important for guiding the FRB-anchored substrate to the kinase active site, and that the P_+2 residue Val391 may be involved in contacts to the kinase C-lobe, where, by analogy with canonical kinases, the peptide segment of the phosphorylation site and its immediate vicinity are expected to bind. c, Superposition of the FRB–S6K1 interface onto the FRB–rapamycin–FKBP12 structure25 (FKBP12 is omitted for clarity), highlighting the similarities in the binding of the Leu396 side chain to the same pocket as rapamycin’s key C23 methyl group and the flanking portions of its triene arm. The FRB–S6K1 interface is coloured as in Fig. 1b; rapamycin is green and its associated FRB domain is cyan. In the crystals of the FRB–S6K1389–414 fusion protein, S6K1 residues 389–391 and 411–414 are disordered, while residues 405–410 are involved in crystal packing. d, Quantification of the reactions shown in Fig. 1h.
e, X-ray data collection and refinement statistics for the FRB2018–2114–S6K1389–414 fusion protein structure.
Extended Data Figure 2 | RAPTOR–4EBP1 TOS interface and density in the cryo-EM structure of mTORC1. a, Human RAPTOR–4EBP1 TOS interface from the 3.0 Å refined human mTORC1 cryo-EM structure, coloured as in Fig. 2b. Only the RAPTOR side chains that make hydrogen bonds (red dotted lines) or electrostatic or van der Waals contacts to 4EBP1 are shown. b, Stereo view of the cryo-EM density of the human RAPTOR–4EBP1 TOS interface. Although the complex contained full-length 4EBP1, only an eight-residue segment is ordered in the cryo-EM reconstruction (also see Extended Data Fig. 7a). c, Cryo-EM data collection and refinement statistics.
Extended Data Figure 3 | See next page for caption.
Extended Data Figure 3 | atRaptor–TOS motif crystal structures and human RAPTOR–TOS motif dissociation constants. a, Stereo view of the mF₀ − dF₀ electron density of the atRaptor–4EBP₁<sup>98–118</sup> co-crystals, calculated with phases from atRaptor before any 4EBP₁ was built into the model. The structure is coloured as in Fig. 2b, and the map, calculated at 3.0 Å and contoured at 2.2σ, is shown as blue mesh. Of 20 residues in the 4EBP₁ peptide in the crystals, only the 8-residue segment shown is ordered. b, Stereo view of the mF₀ − dF₀ electron density, calculated as in a, of the atRaptor–S6K₁<sup>11–14</sup> co-crystals. The 3.1 Å map is contoured at 2.2σ. As with the 4EBP₁ co-crystals, only 8 of the 14 S6K₁ residues are ordered. c, Stereo view of the mF₀ − dF₀ electron density, calculated as in a, of the atRaptor–PRAS40<sup>124–139</sup> co-crystals. The 3.35 Å map is contoured at 1.9σ. Only 8 of the 16 PRAS40 residues are ordered. In addition, side chain of Glu₁₃₃ has poor density and is only tentatively built. d, Molecular surface representation of the atRaptor–TOS-binding groove, coloured according to the electrostatic potential (−6kT to +6kT) calculated without the 4EBP₁ peptide (shown as sticks) using APBS<sup>24</sup> and illustrated with PyMOL. The TOS-binding groove has an overall basic electrostatic potential due to five arginine and one lysine residues, explaining the tendency of acidic residues to be present at the non-conserved and flanking positions of the TOS motif. e, Binding of the indicated human 4EBP₁ TOS mutant peptides (mutation in red) to human RAPTOR measured by fluorescence polarization anisotropy. Graph shows means as dashes and values from three independent experiments with the indicated markers and colours. Dissociation constants from the nonlinear regression fitting of the data are also shown, and simulated binding curves are overlaid on the data. f, Binding of the TOS motif peptides of human 4EBP₁ (blue), S6K₁ (red), and PRAS40 (green) to human RAPTOR measured by fluorescence polarization anisotropy. Graph shows means as dashes and values from three independent experiments with the indicated markers and colours. Also shown are the dissociation constants from the nonlinear regression fitting of the data and the simulated binding curves.
Extended Data Figure 4 | See next page for caption.
Extended Data Figure 4 | PRAS40 is a competitive inhibitor of the FRB substrate-recruitment site and additionally binds to mLST8.

a, Inhibition of mTOR ΔN–mLST8 (30 nM) phosphorylating the TOS-less 4EBP142–64 (10 μM) by indicated PRAS40 fragments (red rectangles mark TOS, β-strand, and amphipathic α-helix). Incorporation of \(^{32}\text{P}\) is plotted as a fraction of the zero PRAS40 reaction of each series, with means as dashes and values from independent experiments with the indicated markers and colours (\(n = 3\) for PRAS40206–256; \(n = 2\) for PRAS40114–256, and PRAS40173–256). IC\(_{50}\) values from the nonlinear regression fitting of the data and their simulated curves are also shown. b, Co-crystal structure of the PRAS40173–256–mTORΔN–mLST8 complex. The mTOR FAT domain is coloured in cyan, FRB in salmon, kinase in pink, mLST8 in green, and PRAS40 in red. c, Anomalous diffraction Fourier map (red mesh) of crystals containing the SeMet-substituted L225M mutant of PRAS40206–256 bound to mTOR ΔN–mLST8 showing two SeMet peaks that confirm the direction of the PRAS40 helix. The 5.4 Å map is contoured at 3.5\(σ\) and superimposed on the structure of the wild-type complex, which has a slightly different unit cell from the SeMet crystals. The anomalous diffraction map of wild-type selenomethionine-substituted PRAS40 co-crystals is shown in the main text (Fig. 3b). d, The m\(F_o\) – d\(F_c\) electron density of PRAS40114–207, which contains only one of the three phenylalanine residues present in the PRAS40173–256 polypeptide, bound to mTOR ΔN–mLST8, confirming the sequence assignment of the PRAS40 β-strand. The map was calculated with phases from before PRAS40 was built into the model. The structure and map, calculated at 3.0 Å and contoured at 2\(σ\), are coloured as in Fig. 3d. e, Phosphorylation of full-length 4EBP1 by apo-mTORC1 (left) does not obey Michaelis–Menten kinetics, and in the absence of a single substrate \(K_m\) value we cannot calculate the \(K_i\) of full-length PRAS40 for the reaction of Fig. 3e. Left: the \(^{32}\text{P}\) incorporation data, plotted as velocity over enzyme concentration (means ± s.d., \(n = 11\) except for the 10, 50, and 200 μM reactions, which are \(n = 6\), for the curve of apo-mTORC1–phosphorylating full-length 4EBP1. The curve of reaction velocity/[enzyme] versus substrate concentration is parabolic, with product levels comparatively higher at low substrate concentrations (up to around 10 μM) and lower at higher substrate concentrations than a Michaelis–Menten-type response. The two substrate concentration ranges display very different \(K_m\) and \(k_{cat}\) values. The entire substrate range can thus be modelled (black curve) as the sum of two reactions, one having a tight \(K_m\) of approximately 2 μM but very slow \(k_{cat}\) of 0.003 s\(^{-1}\) (blue dashed curve), and another reaction with a weak \(K_m\) of 545 μM but a faster \(k_{cat}\) of 0.065 s\(^{-1}\) (green dashed curve). The tight \(K_m\) reaction is dependent on the TOS motif, as its mutation (right; graph shows means as dashes and values from three independent experiments) results in a reaction that obeys Michaelis–Menten kinetics, with \(K_m\) and \(k_{cat}\) values of 462 μM and 0.053 s\(^{-1}\), respectively, which are very similar to the values of the weak \(K_m\) faster \(k_{cat}\) curve of wild-type 4EBP1. We presume that the TOS-independent weak \(K_m\) reaction reflects, in part, substrate interactions with the FRB. It is possible that the non-Michaelis–Menten behaviour is due to the presence of multiple, probably non-equivalent phosphorylation sites on 4EBP1. We also cannot reliably measure the \(K_m\) value of full-length S6K1, as this substrate aggregates and then precipitates at concentrations higher than approximately 50 μM, before reaching saturation (not shown). f, X-ray data collection and refinement statistics for the structures of mTOR ΔN–mLST8 bound to PRAS40 fragments.
Extended Data Figure 5 | See next page for caption.
Extended Data Figure 5 | Cryo-EM reconstruction of mTORC1 and RHEB–mTORC1 complexes. 

**a** Flow chart of single particle cryo-EM data processing. Details are described in Methods. 

**b** Left: gold-standard FSC plots between two independently refined half-maps for the consensus mTORC1 C2 reconstruction (red curve), the masked monomer with signal subtraction (blue curve), the RHEB–mTORC1 C2 reconstruction (purple curve), and the masked RHEB–mTORC1 monomer with signal subtraction containing additional signal-subtracted particles from a second, down-scaled dataset (green curve). The FSC cutoff of 0.143 and associated resolution for each plot are marked. Right: gold-standard FSC plots for the three focused refinements of the consensus mTORC1 C2 reconstruction with mask 1 (purple curve), mask 2 (red curve), and mask 3 (green curve) described in Methods. Also shown are the corresponding curves for RHEB–mTORC1 masks 1, 2, 3 in cyan, orange, and pink, respectively. The FSC cutoff of 0.143 and associated resolution ranges of the three focused refinements for consensus mTORC1 and RHEB–mTORC1 are marked on each plot. 

**c** The four largest RHEB-less 3D classes of the flowchart (a) superimposed on the C-lobe of the kinase domain (marked by arrow) of one mTOR protomer. The closest approach of the two mLST8 subunits ranges from 123 Å (‘closed’ conformation in light blue) to 145 Å (‘open’ conformation in pink). The RHEB-containing class and two minor classes of suboptimal density are omitted for clarity. The four classes shown seem to represent samples along a continuum of conformations between the open and closed states, as more intermediate states get populated in a 3D classification with a larger number of classes (not shown). The 3D classes shown are from a calculation with a partial dataset approximately 50% the size of the final dataset.

**d** Comparison of the RHEB-containing class (green, with the RHEB density in red) with the RHEB-less classes (coloured light grey to dark grey). The maps are superimposed as in c. The RHEB-containing class has two RHEB molecules with very similar density, even though the 3D classification was done in C1. In the figure, one of the two RHEBs is occluded in the left and right panels, and is in lighter background (labelled as RHEB-2) in the middle panel. None of the RHEB-less classes has any significant density at either of the two RHEB-binding sites. The RHEB-containing class has an inter-mLST8 distance intermediate between the open and closed conformations of the RHEB-less classes, but the relative positions of N-heat and its associated RAPTOR are distinct from the apparent continuum of conformational states of the RHEB-less classes. Curved arrows indicate the transitions from the RHEB-less classes (‘minus’ sign) to the RHEB-containing (‘plus’ sign) class. 

**e** Cryo-EM density of the RHEB-containing particles from the 3D classification showing cartoon representations of the refined RHEB–GTPγS structure (yellow, its switch I and II segments in red), and the RHEB-interacting portions of the mTOR N-heat (green), M-heat (pink), and FAT (pink) segments. 

**f** FSC plots of the final model versus the composite cryo-EM map from REFMAC5 (black), and of a model validation protocol refining against one of two half-maps after an initial random displacement of atoms is applied to the model to remove model bias (FSCwork in red), and cross-validating the same model against the other half-map (FSCfree green).
Extended Data Figure 6 | Secondary structure and conservation of mTOR. Human mTOR sequence showing conservation from yeast to man (blue column graph above sequence) and secondary structure elements in the refined model. Helices are indicated as rectangles, \( \beta \)-strands as arrows, segments lacking regular secondary structure as solid lines, and disordered regions as dashed lines. N-heat secondary structure is coloured green, M-heat in orange, FAT in cyan (including helices \( f_{1α1}–f_{1α6} \), which are continuous with the FAT structure, even though they are outside the FAT boundary defined by sequence conservation in PIKKs), the kinase domain N-lobe in yellow (FRB helices are named \( kf_{α1}–kf_{α4} \) for consistency with the \( mTOR^\Delta N \)-mLST8 paper), and C-lobe in pink. The three hatched N-heat helices have not been assigned a sequence, and their boundaries are indicated tentatively. The dashed lines indicate disordered regions.
Extended Data Figure 7 | See next page for caption.
Extended Data Figure 7 | Apo-mTORC1 cryo-EM density, interfaces, and conformational flexibility. 

**a.** The 4EBP1 amphipathic helix density on the FRB does not have interpretable cryo-EM density. The map shown is of a 3D class (~30% of particles) with the highest relative level of density; the FRB side chains that contact the S6K1 substrate in Fig. 1c are shown as red sticks to map the site, the rest of mTOR is coloured as in Fig. 4a, and AMPPNP is in space-filling representation. Unlike the 4EBP1 TOS, which has a level of density comparable to that of its binding site (Extended Data Fig. 2b), the putative density of the 4EBP1 amphipathic helix is much weaker than that of the FRB, suggestive of partial occupancy. We presume this is due, in part, to our cryo-EM samples containing 260 mM NaCl, which substantially reduces 4EBP1 phosphorylation and thus probably further weakens substrate-FRB association (right).

**b.** Stereo view of the 3.0 Å cryo-EM density of the consensus apo-mTORC1 reconstruction, showing mTOR N-heat (residues approximately 650–850 shown in stick representation coloured green, red, and blue for C, O, and N atoms, respectively). 

**c.** Stereo view of the 3.0 Å cryo-EM density of the consensus apo-mTORC1 reconstruction showing mTOR M-heat (residues approximately 960–1105 shown in stick representation coloured sand, red, and blue for C, O, and N atoms, respectively). 

**d.** The end of the N-heat solenoid (residues 848–898; green) is anchored on the middle of the FAT domain (residues 1565–1627; light cyan). Close-up view showing side chains (glycine Cα atoms as spheres) and backbone groups (blue spheres for amide and sticks for carbonyl groups) involved in intra-molecular van der Waals or hydrogen bonds (yellow dotted lines). For clarity, only N-heat residues 836–903 and FAT residues 1537–1664 are shown. 

**e.** The mTOR binding elements of RAPTOR (purple) are encompassed within its conserved RNC (raptor N-terminal conserved)7, with the caspase domain contacting M-heat of one mTOR protomer (sand), the caspase insertion contacting both M-heat and the N-heat (green) of the other mTOR protomer, and the first three armadillo repeats of its solenoid contacting N-heat. Side chains are shown as in d. Hydrogen bond contacts are shown as red dotted lines. N-heat and M-heat structural elements above the plane of the figure are omitted for clarity.

**f.** The conformational flexibility of apo-mTORC1 is associated with bending at a major hinge region of three heat repeats (indicated by a box) in the N-heat solenoid, in between its FAT and RAPTOR–M-heat interacting segments. Figure shows Cα trace of the tripartite interface between N-heat of protomer 2, M-heat of protomer 1, and RAPTOR of the four apo-mTORC1 3D classes of Extended Data Fig. 5c (coloured as in Fig. 4a) and RHEB–mTORC1 (all red). The four apo-mTORC1 classes were refined to 4.5 Å or better, and together with RHEB–mTORC1 were superimposed on RAPTOR (residues 52–422). Figure also highlights the flexibility on the N-terminal half of the N-heat solenoid in apo-mTORC1 (see Supplementary Discussion).
Extended Data Figure 8 | See next page for caption.
Extended Data Figure 8 | RHEB-induced conformational change in mTORC1. a, Stereo view of the cryo-EM density from the 3.4 Å RHEB–mTORC1 reconstruction, showing the RHEB–mTOR interface in the same orientation and colouring as in Fig. 4c. The RHEB-interacting segments of mTOR are nα3–nα7 of N-heat, mα2–mα4 of M-heat, and fα2–fα3 of FAT. Most of the contacts made by RHEB are from its switch I and switch II regions, with a small number of additional contacts to N-heat and M-heat contributed by the nearby segments of residues 5–7 and 106–111. b, Stereo view of cryo-EM density from RHEB–mTORC1 (sand) and the 3.0 Å apo-mTORC1 (green), with the two structures and maps superimposed on the C-lobes as in Fig. 5d. AMPPNP (orange) cryo-EM density of apo-mTORC1. d, Steady-state kinetic analysis of mTORC1 phosphorylation of intact 4EBP1 in the presence of 250 μM RHEB–GTPγS. Reactions quantified by 32P incorporation and plotted as velocity over enzyme concentration (means as dashes and values from two independent experiments as filled circles). The Kₘ and kₗc values, calculated by nonlinear regression fitting of the data, and simulated curves are also shown. Note that in contrast to the reaction in the absence of RHEB shown in Extended Data Fig. 4e, the curve of reaction velocity versus 4EBP1 concentration obeys Michaelis–Menten kinetics.

e, RHEB–GTPγS activation of 4EBP1 phosphorylation by mTORC1 under single-turnover conditions. A master mix of excess mTORC1 (500 nM) over 4EBP1 substrate (100 nM) was incubated with 250 μM RHEB–GTPγS or 250 μM RHEB–GDP in the standard kinase buffer on ice for 5 min. Reactions were started by the addition of a mixture of cold ATP (50 μM final) and [γ-32P]ATP (8 μCi per reaction time point). The reactions were done on ice to slow them down. At the indicated time points, an aliquot of the reaction was drawn, stopped, and analysed as described in Methods. The experiment was repeated three times with very similar results. f, ATP steady-state kinetic parameters of ATP hydrolysis by mTORC1 in the presence of 250 μM RHEB–GDP (left, blue plot) or RHEB–GTPγS (right, red plot). Reactions were quantified by 32P incorporation as in d. Graph shows means as dashes and values from three independent experiments with the indicated markers and colours. The steady-state kinetic constants of the RHEB–GDP-containing reaction are approximate owing to the weak signal of these reactions. g, As expected, RHEB–GTPγS did not activate the truncated mTORΔN–mLST8 complex phosphorylating 4EBP1 or S6K1367–404 (10 μM both; mTORΔN at 20 and 30 nM, respectively) (left; experiments were repeated twice with very similar results). mTORΔN–mLST8 has an intermediate kₗc of 0.66 s⁻¹ (Extended Data Fig. 1b) compared with the 0.09 and 2.9 s⁻¹ kₗc values of apo-mTORC1 and RHEB–mTORC1, respectively (Fig. 5g). mTORΔN–mLST8 has a distinct FAT conformation, probably because of the absence of N-heat. Right: superposition of the FAT plus kinase domain portions of inactive apo-mTORC1 on the crystal structure of mTORΔN–mLST8 done by aligning their C-lobes. Apo-mTORC1 is in green and mTORΔN is coloured blue for FAT, yellow for N-lobe, and pink for C-lobe. The rotation axes (red lines) are numbered according to the hinges of Fig. 5c. Compared with the inactive-to-active transition, the comparison of the mTORΔN FAT conformation with that of the inactive state shows bigger changes around the major hinge with a rotation in the opposite direction and a different rotation axis far from the hinge axis (labelled ‘1’). The rotations around the two minor hinges are comparably modest although distinct, with the rotation axes nearly orthogonal to those of the inactive-to-active transition. h, Autoradiogram showing activation of mTORC1 phosphorylating 4EBP1 (10 μM) by RHEB–GTPγS, repeated three times. Gel quantification is shown in Fig. 5b. i, Steady-state kinetic analysis of mTORC1 phosphorylating S6K1367–404 in the presence of 250 μM RHEB–GDP (top row) or RHEB–GTPγS (bottom row). Incorporation of 32P data are plotted as velocity over enzyme concentration in Fig. 5g (n = 3).
Extended Data Figure 9 | See next page for caption.
Extended Data Figure 9 | Hyperactivating mTOR mutations cluster in three regions of the mTOR structure. a, Hyperactivating mutations that cluster at the major hinge region of the FAT domain. The mutations, which occur at residues with structure-stabilizing roles, probably disrupt the structural integrity of the FAT clamp and thus its ability to block the movement of the N-lobe into the active position. Cancer-genome mutations shown experimentally to be hyperactivating are mapped onto the apo-mTORC1 (left) and RHEB–mTORC1 (right) structures. The two structures are aligned on the C-lobes of their kinase domains to highlight the different packing arrangements at the hinge. The side chains of mutated residues are indicated with the letter ‘M’ and coloured dark cyan, the side chains that they interact with are in light cyan, while the rest of the structural elements are coloured as in Fig. 4a. Red dotted lines indicate groups within hydrogen bond distance. b, The largest cluster of hyperactivating mutations is centred on an N-lobe helical extension (kα3, kα3b) that is anchored in a pocket between the C-lobe and FAT domains. Here, the structural mutations either at the N-lobe kα3–kα3b helices or at their binding site on the C-lobe and the adjacent FAT domain would weaken the structural coupling of the N-lobe with the C-lobe, possibly allowing the N-lobe to assume conformations closer to its active state. Mutations mapped onto the apo-mTORC1 (left) and RHEB–mTORC1 (right) structures. c, Hyperactivating mutations that cluster where the FAT transitions into and packs with the N-lobe. Mutations here probably destabilize the structural elements and their packing which prevents the N-lobe from moving into its active position, mimicking the RHEB-induced conformational change and the associated looser FAT–N-lobe interface. Mutations are mapped onto apo-mTORC1 (FAT in cyan; N-lobe in yellow, C-lobe in pink) and RHEB–mTORC1 (all in grey). The two structures are superimposed on their N-lobe domains to facilitate comparison. d, Steady-state kinetic analysis of S6K1 367–404 phosphorylation by 30 nM mTORC1 containing wild-type or the indicated hyperactive mTOR mutants. Incorporation of 32P was quantified and velocity over enzyme concentration values were plotted as means (dashes) and values from two independent experiments with the indicated markers and colours. Dissociation constants from the nonlinear regression fitting of the data are also shown, and simulated binding curves are overlaid on the data.
Extended Data Table 1 | Data collection and refinement statistics for the atRaptor–TOS and apo-atRaptor crystal structures

| Data collection | atRaptor | atRaptor-4EBP1<sup>99-110</sup> | atRaptor-S6K1<sup>1-146</sup> | atRaptor-PRAS40<sup>124-139</sup> |
|-----------------|----------|-------------------------------|-----------------------------|----------------------------------|
| Space group     | P<sub>2</sub>1<sub>1</sub> | P<sub>2</sub>1<sub>1</sub> | P<sub>2</sub>1<sub>1</sub> | P<sub>2</sub>1<sub>1</sub> |
| Cell dimensions | 89.1, 112.6, 134.1 | 89.1, 113.1, 153.5 | 89.1, 113.1, 152.9 | 89.1, 113.1, 151.8 |
| α, β, γ (°)     | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 |
| Resolution (Å)  | 50-3.00 | 80-3.00 | 80-3.10 | 80-3.35 |
| R<sub>meas</sub> | (3.11-3.00) | (3.11-3.00) | (3.21-3.10) | (3.47-3.35) |
| R<sub>free</sub> | 0.086 (0.533) | 0.082 (0.800) | 0.084 (0.819) | 0.088 (0.758) |
| R<sub>i/f</sub>  | 0.057 (0.362) | 0.035 (0.307) | 0.035 (0.331) | 0.042 (0.377) |
| R<sub>i/f</sub>  | 19.8 (2.4) | 19 (1.53) | 19.2 (1.76) | 22.3 (2.3) |
| CC<sub>i/f</sub> | (0.713) | (0.801) | (0.846) | (0.770) |
| Completeness (%)| 97.4 (97.3) | 98.7 (99.6) | 99.2 (99.4) | 98.5 (97.6) |
| Redundancy      | 3.1 (3.0) | 6.2 (7.2) | 6.5 (6.9) | 5.1 (4.8) |

Refinement

| Resolution (Å) | 20.3-0 | 20.3-0 | 20.3-11 | 20.3-35 |
| No. reflections | 24264 | 27720 | 25050 | 19535 |
| R<sub>i/f</sub> / R<sub>free</sub> (%) | 24.4 / 27.8 | 22.8 / 27.4 | 21.6 / 25.6 | 21.0 / 26.5 |
| No. atoms      | Protein 8310 | 8378 | 8332 | 8331 |
|               | Ligand/ion 0 | 0 | 0 | 0 |
|               | Water 0 | 0 | 0 | 0 |
| B factors      | Protein 75.3 | 102.4 | 104.4 | 115.3 |
|               | Ligand/ion - | - | - | - |
|               | Water - | - | - | - |
| R.m.s. deviations | Bond lengths (Å) 0.008 | 0.008 | 0.008 | 0.008 |
|               | Bond angles (°) 1.356 | 1.371 | 1.348 | 1.355 |

*Values in parentheses are for highest-resolution shell. All datasets were collected from a single crystal each.*
Life Sciences Reporting Summary

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Experimental design

1. Sample size

Describe how sample size was determined. No sample size calculations were performed.

2. Data exclusions

Describe any data exclusions. No data were excluded from analyses.

3. Replication

Describe whether the experimental findings were reliably reproduced. All attempts at replication were successful.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups. Randomization was used only in structure refinement (Rfree) and cryoEM refinement (RELION) and it was random.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis. No blinding was used.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or the Methods section if additional space is needed).

| n/a | Confirmed |
|-----|-----------|
|     | ✓ The exact sample size \( n \) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
|     | ✓ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly. |
|     | ✓ A statement indicating how many times each experiment was replicated |
|     | ✓ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section) |
|     | ✓ A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
|     | ✓ The test results (e.g. \( p \) values) given as exact values whenever possible and with confidence intervals noted |
|     | ✓ A summary of the descriptive statistics, including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
|     | ✓ Clearly defined error bars |

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study. ImageGauge (version 4.1) was used to quantify data from kinase and ATP hydrolysis assays. Analyses of biochemical data were performed using...
Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All unique materials used are readily available.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

1. Cell Signaling Cat#9205 Lot#16 Phospho-p70 S6 Kinase (Thr389) antibody produced in rabbit

Validation statement on vendor’s website: Phospho-p70 S6 Kinase (Thr389) Antibody detects endogenous levels of p70 S6 kinase only when phosphorylated at threonine 389. This antibody also detects p85 S6 kinase when phosphorylated at the analogous site (Thr412), and possibly S6KII phosphorylated at Thr388...Polyclonal antibodies are produced by immunizing animals with a synthetic phosphopeptide corresponding to residues around Thr389 of human p70 S6 kinase. Antibodies are purified by protein A and peptide affinity chromatography.

2. Cell Signaling Cat#7074 Lot#22 Anti-rabbit IgG, HRP-linked Antibody

Validation statement on vendor’s website: Designed for use with rabbit polyclonal and monoclonal antibodies, this affinity purified goat anti-rabbit IgG (heavy and light chain) antibody is conjugated to horseradish peroxidase (HRP) for chemiluminescent detection. This product is thoroughly validated with CST primary antibodies and will work optimally with the CST western immunoblotting protocol, ensuring accurate and reproducible results.

3. Sigma Aldrich Cat#A8592 Lot#SLBD9930 Monoclonal ANTI-FLAG® M2-Peroxidase (HRP) antibody produced in mouse

Validation statement on vendor’s website: The Monoclonal ANTI-FLAG M2-Peroxidase is a mouse IgG antibody covalently conjugated to horseradish peroxidase (HRP). The antibody binds to FLAG fusion proteins and recognizes the FLAG epitope at N-terminal, Met-N-terminal, C-terminal, and internal FLAG peptides. Applications for the conjugate include Western blots, dot blots, ELISA, and immunocytochemistry.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

HEK-293F cells used for protein overexpression and in vivo assays were purchased from Invitrogen.

b. Describe the method of cell line authentication used.

Vendor authenticated.

c. Report whether the cell lines were tested for mycoplasma contamination.

Cell lines were not tested for mycoplasma contamination.

d. If any of the cell lines used in the paper are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used.
Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals
   Provide details on animals and/or animal-derived materials used in the study.
   No animals were used in the study.

Policy information about studies involving human research participants

12. Description of human research participants
   Describe the covariate-relevant population characteristics of the human research participants.
   The study did not involve human research participants.