mTOR kinase structure, mechanism and regulation

Haijuan Yang1, Derek G. Rudge1, Joseph D. Koos1, Bhamini Vaidiallingam1, Hyo J. Yang1 & Nikola P. Pavletich1,2

The mammalian target of rapamycin (mTOR), a phosphoinositide 3-kinase–related protein kinase, controls cell growth in response to nutrients and growth factors and is frequently deregulated in cancer. Here we report co-crystal structures of a complex of truncated mTOR and mammalian lethal with SEC13 protein 8 (mLST8) with an ATP transition state mimic and with ATP-site inhibitors. The structures reveal an intrinsically active kinase conformation, with catalytic residues and a catalytic mechanism remarkably similar to canonical protein kinases. The active site is highly recessed owing to the FKBP12–rapamycin–binding (FRB) domain and an inhibitory helix protruding from the catalytic cleft. mTOR–activating mutations map to the structural framework that holds these elements in place, indicating that the kinase is controlled by restricted access. In vitro biochemistry shows that the FRB domain acts as a gatekeeper, with its rapamycin–binding site interacting with substrates to grant them access to the restricted active site. Rapamycin–FKBP12 inhibits the kinase by directly blocking substrate recruitment and by further restricting active-site access. The structures also reveal active-site residues and conformational changes that underlie inhibitor potency and specificity.

The mTOR pathway controls cell growth in response to energy, nutrients, growth factors and other environmental cues, and it figures prominently in cancer1,2. Central to the pathway is the mTOR protein, which belongs to the phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family3. mTOR assembles into two complexes with distinct inputs and downstream effects. mTOR complex 1 (mTORC1) is defined by its RAPTOR subunit4–6, which is replaced by RICTOR in mTOR complex 2 (mTORC2). Both complexes also contain the requisite mLST8 subunit7,8, but they differ in a number of other subunits that interact with RAPTOR or RICTOR1.

mTORC1 regulates cell growth by promoting translation, ribosome biogenesis and autophagy1,4,9. Its activation requires nutrients and amino acids, which result in the RAPTOR-mediated recruitment of mTORC1 to lysosomes and late endosomes10,11, and co-localization with its activator, the small GTPase RHEB12,13. Ways in which RHEB is proposed to act include binding to and activating the mTOR kinase domain14, and displacing the mTORC1 inhibitor PRAS40 from RAPTOR15,16. RHEB in turn is negatively regulated by the GTPase-activating domain of the TSC2 tumour suppressor, which relays signals from multiple growth factor and stress pathways17. mTORC1 substrates include the eIF4E-binding protein 1 (4E-BP1) and ribosomal S6 kinases (S6K), which control cap-dependent translation initiation and elongation, respectively18. Phosphorylation of 4E-BP1 and S6K1 is dependent on their Tor signalling sequence (TOS) motif19,20, with binding to RAPTOR and is also present in the negative regulator PRAS4017,22.

mTORC2 responds primarily to growth factors, promoting cell cycle entry, cell survival, actin cytoskeleton polarization and anabolic output23,24. Its substrates include the Ser/Thr protein kinases AKT, SGK and PKC, which share the hydrophobic motif phosphorylation site with S6K12,5. Rapamycin, which forms a ternary complex with the FK506-binding protein 12 (FKBP12) and the FRB domain of mTOR, is thought to be an allosteric inhibitor25,26. Rapamycin–FKBP12 inhibits mTORC1 to a variable extent that is substrate and phosphorylation-site dependent27, and it does not bind to mTORC228. To overcome these limitations, ATP-competitive inhibitors that potently and uniformly inhibit both mTORC1 and mTORC2 are being developed as anticancer agents29.

The six mammalian PIKKs regulate diverse cellular processes30. They share three regions of homology consisting of a ~600-residue FAT domain (named after FRAP, ATM and TRRAP), a ~300-residue PIKK catalytic domain and a ~35-residue FATC domain at the carboxy terminus28. In mTOR, the ~100-residue FRB domain is thought to occur between the FAT domain and the catalytic domain, and the region amino terminal to the FAT domain is required for binding to RAPTOR or RICTOR1.

Here we present the crystal structure at 3.2 Å of a ~1,500-aminocid mTOR–mLST8 complex containing the FAT, FRB, kinase and FATC domains, as well as the structures of this complex bound to an ATP transition state analogue and, respectively, ATP-competitive inhibitors. We discuss their implications for understanding mTOR function, regulation and inhibition by rapamycin and ATP-competitive compounds.

Overall structure of mTORN–mLST8

Crystals were grown using N-terminally truncated human mTOR (residues 1376–2549; hereafter mTORN) bound to full-length human mLST8 (Supplementary Table 1). The complex was produced in an HEK293-F cell line that was stably transfected sequentially by Flag-tagged mLST8 and Flag-tagged mTORN vectors. The kinase activity of mTORN–mLST8 is overall comparable to that of mTORC1 (Supplementary Fig. 1). mTORC1 is more active towards low-micromolar concentrations of S6K122 (kinase-inactive mutant) and 4E-BP1, consistent with RAPTOR recruiting these substrates through their TOS motifs, whereas mTORN–mLST8 is more active at higher substrate concentrations.

The mTORN–mLST8 structure has a compact shape (Fig. 1). The FAT domain, which consists of ~10 helical repeats, forms a C-shaped α helix that wraps halfway around the kinase domain and clamps onto it. mLST8 and the FRB domain protrude from the kinase domain,

1Structural Biology Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10065, USA. 2Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, New York 10065, USA.
on opposite sides of the catalytic cleft. The FATC domain is integral to the kinase domain structure.

The ~550-residue mTOR kinase domain adopts the two-lobe structure that is characteristic of both the PI3K and canonical protein kinase families\(^{29}\). It consists of an N-terminal lobe (N lobe), a larger C-terminal lobe (C lobe) and a cleft between the two that binds to ATP. The mTOR kinase domain contains the core PI3K kinase domain fold, but with substantial differences. Whereas PI3K kinase domain structures superimpose essentially across their entire ~350 residues, only ~250 residues of the mTOR kinase domain superimpose on PI3Ks (Supplementary Table 2). In addition, the mTOR kinase domain contains ~200 additional residues of insertions that decorate the common fold. The largest of these is the FRB domain (residues 2021–2118), which is inserted within the kinase N lobe (Fig. 1). There is also a ~40-residue insertion in the C lobe that forms the binding site for mLST8 (residues 2258–2296; hereafter LBE).

**Kinase domain structure**

The mTOR kinase domain structure starts before the FRB domain, with the long k\(^{2}\) helix (k denoting kinase domain) that is present in PI3Ks as well (Fig. 2a and Supplementary Fig. 2a). The k\(^{2}\) helix is integral to the structure of the N lobe, because it packs in the concave surface of the N-lobe \(\beta\)-sheet in both mTOR and PI3Ks\(^{29}\). The FRB insertion occurs immediately after the k\(^{2}\) helix. It is followed by a \(\beta\)-strand and two short helices that pack with the base of the FRB and replace the k\(^{2}\) helix of the PI3K kinase domain fold. Thereafter, the mTOR and PI3K N lobes have similar structures.

The mTOR structure indicates that the k\(^{2}\) helix is present in all PIKKs (Supplementary Fig. 3a). The SMG1, DNA-PKcs and TRRAP PIKKs also seem to have an FRB-like domain, because they respectively contain insertions of 128, 95 and 128 residues that are predicted to be \(\alpha\)-helical. This is supported by the 6.6 Å X-ray diffraction data of DNA-PKcs reported recently\(^{30}\). Although the deposited DNA-PKcs model\(^{30}\), which contains a PI3K-derived kinase domain, lacks the FRB insertion, a reinterpretation of the data using the mTOR structure reveals electron density indicative of an FRB-like four-helix bundle (Supplementary Fig. 3b). ATM and ATR seem to lack a comparable insertion.

In the C lobe, the vicinity of the catalytic cleft contains four structural insertions in the PI3K core structure (LBE, k\(^{2}\) AL, k\(^{2}\) 9b and FATC; Fig. 2a). These form a spine of interactions centred on the activation loop, which is a ~30 residue segment with a central role in the function and regulation of canonical protein kinases. It forms part of the polypeptide-binding site, provides an active-site residue and undergoes a conformational change, typically a disorder-to-order transition, on kinase activation\(^{31}\). The activation loop is thought to have an analogous role in PI3Ks, among which it is ordered in only the class III PIK3C3 structures\(^{22}\). In the mTOR structure, the entire activation loop is well ordered (Supplementary Fig. 2b) and its middle portion contains the k\(^{2}\) AL helix insertion. One side of the activation loop packs with the k\(^{2}\) 9b insertion, and the other side packs with the FATC (Fig. 2a). The FATC is not unique to PIKKs in its entirety, because its N-terminal half forms a helix (k\(^{11}\)) that is also present in PI3K structures\(^{29,32}\). Its C-terminal half, which is absent from PI3Ks, forms three short helices that pack with the activation loop on one side and with the LBE on the other (Fig. 2a).

The interactions that the FATC makes with the activation loop suggest that it may have a role in stabilizing the activation loop structure, and the LBE, through its interactions with the FATC, may contribute to this indirectly (Supplementary Fig. 4a). The FATC and activation loop sequences are highly conserved among PIKKs. Although the LBE is not conserved, all PIKK family members contain an LBE-like insertion that may similarly pack with FATC (Supplementary Fig. 3a).

The k\(^{2}\) 9b insertion (residues 2425–2436) plugs one end of the catalytic cleft (Fig. 2a). It partly overlaps with a segment, called the negative regulatory domain (residues 2430–2450), whose deletion activates mTOR in vitro\(^{44,45}\) and in vivo\(^{46,47}\). After k\(^{2}\) 9b, there is a 55-residue unstructured segment (residues 2437–2491) that is not conserved and has variable length in mTOR orthologues (Supplementary Fig. 2a).

**Active-site conformation**

To assess the activation state of the kinase domain structure and investigate the mechanism of phosphotransfer, we co-crystallized mTOR\(^{AN}\)–mLST8 with ADP, Mg\(^{2+}\) and MgF\(_{3}^{-}\), a mimic of the \(\gamma\)-phosphate group of ATP in the transition state\(^{38}\) (Supplementary Table 1). The \(F_{o} - F_{c}\) map of these crystals at 3.5 Å shows strong electron density extending from the \(\beta\)-phosphate group of ADP, consistent with a bound MgF\(_{3}^{-}\} group (Fig. 2b). The presence of two additional Mg\(^{2+}\} ions is supported by the anomalous dispersion maps of apo-crystals soaked in AMPPPN-Mg\(^{2+}\} (Supplementary Fig. 4b).

A superposition with the protein kinase CDK2 bound to the same ATP transition state mimic\(^{36}\} reveals that the arrangement of key active-site residues and MgF\(_{3}^{-}\} is remarkably conserved in mTOR (Fig. 2c and Supplementary Fig. 4c). These include the C-lobe Asn 2343 and Asp 2357, which in both mTOR and protein kinases serve as metal ligands, and Asp 2338, which in protein kinases has a key catalytic role in orienting and activating the substrate hydroxyl group for nucleophilic attack\(^{36,37}\}. The mTOR–CDK2 superposition also reveals a coincidence of the mTOR His 2340 and CDK2 Lys 129 side chains (Fig. 2c). In protein kinases, a basic residue at this position interacts with both the substrate hydroxyl group and the \(\gamma\)-phosphate transition state mimic, and is postulated to stabilize the build-up of charge at the transition state\(^{36,37}\}. Consistent with a similar role, we find that the kinase activity of the His 2340Ala mTOR\(^{AN}\)–mLST8 mutant is barely detectable, and comparable to the Asp 2338Ala mutant previously shown to be inactive\(^{36}\} (Supplementary Fig. 4d).

These findings strongly suggest that the crystallized mTOR\(^{AN}\}–mLST8 complex is intrinsically active in the absence of any additional regulatory subunits. They also indicate that PIKKs, and most probably PI3Ks, use the same catalytic mechanism as canonical protein kinases, in contrast to a recently proposed PI3K mechanism\(^{38}\} (Supplementary Fig. 4c).

**Substrate-binding site**

The MgF\(_{3}^{-}\}\gamma\)-phosphate mimic points to an extended C-lobe groove that is highly conserved and is the likely site of substrate binding.
Figure 2 | mTOR kinase domain and active site conformation. a, Superposition of the kinase domains of mTOR and PIK3C3 (Protein Data Bank (PDB) ID, 3IHY; green) in two views rotated by −180°. Left view is related to that of Fig. 1. The FRB is coloured red, and the remaining mTOR insertions are coloured dark blue. The kα2b helix is in the activation loop (A-loop). The black dashed lines delineate the LBE, and the blue dotted loops indicate the disordered region between kα2b and kα11. b, The 3.5 Å Fobs − Fcalc electron density, contoured at 2.5σ, of the mTOR transition state complex before ADP-MgF3-Mg2 ion was built. ADP-MgF3 is shown as sticks and Mg2 ions are shown as spheres. c, Superposition of the mTOR and CDK2 transition state (PDB ID, 3QHW) complexes. CDK2 and its nucleotide are coloured green (its residue labels are shown in parentheses). d, Molecular surface representation of the C-lobe portion of the mTOR catalytic cleft, coloured according to conservation entropy (red invariant in 22 orthologues, orange in 21, yellow-orange in 20 and yellow in 18 or 19). Dashed lines delineate the boundaries of the labelled structural elements. The docked substrate peptide is coloured light blue, with its threonine phosphorylation site shown as sticks, the remaining side chains as spheres (Cβ atoms) and its +1 position indicated by a white arrow.

Figure 2d illustrates this with a CDK2-bound substrate docked by superposing the mTOR and CDK2 transition state complexes. The groove consists of the activation loop, as in protein kinases, but also of portions of the FATC and LBE.

Carboxy-terminal to the phosphorylation site (the + direction), the groove extends only to the +1 position, abruptly terminating with the kα2b helix that plugs this end of the cleft (Fig. 2d). Thereafter, the peptide can exit along the C-lobe towards mLST8 or along the N lobe towards the FRB. The +1 portion of the groove has a pronounced pocket lined with three aromatic residues, consistent with the two classes of mTOR substrates—exemplified by 4E-BP1 and S6K1—that have a proline and a tyrosine residue, respectively, at +1 (Supplementary Fig. 5a, b). The groove extends considerably more in the direction N-terminal to the phosphorylation site (the − direction), and it may account for the low-level sequence preference at −4 and −5 suggested by a positional scanning peptide array.

Restricted access to the active site
The four-helix FRB substantially extends the N-lobe side of the catalytic cleft, whereas the LBE and mLST8 extend the C-lobe side. This gives the cleft a deep, V-shaped cross-section, restricting access to the substrate-binding site at the bottom of the cleft (Fig. 3a). Substrate access is further hindered by one end of the cleft being plugged by kα2b and the 55-residue unstructured segment that follows the helix. Portions of kα9b and the unstructured segment form the negative regulatory region, suggesting that restricted access to the active site negatively regulates mTOR. Helix kα9b is probably the key element because deleting the bulk of the unstructured segment (residues 2443–2486) does not activate mTOR (Supplementary Fig. 6).

The end of the cleft opposite kα9b is unencumbered in the mTOR–mLST8 structure. However, it is near the FAT N terminus, and the region deleted from the crystallized mTOR may restrict cleft access directly or through its associated RAPTOR or RICTOR proteins, or by both means. In support, we find that towards TOS mutant 4E-BP1 and S6K1 substrates, mTOR–mLST8 is more active than mTOR–mLST8, which in turn is more active than mTOR–mLST8–RAPTOR (Supplementary Fig. 6). A restricted active site would reduce the probability of substrates hitting the active site by random diffusion, and could thus be important in allowing for the regulation of phosphorylation by substrate recruitment.

Inhibition by rapamycin–FKBP12
In a model constructed by superposing the FRB domains of the FRB–rapamycin–FKBP12 and mTOR–mLST8 structures, FKBP12 extends from the FRB towards mLST8 on the C lobe, nearly capping the catalytic cleft (Fig. 3b). At their closest approach, FKBP12 and mLST8 are only 8 Å apart, with the catalytic centre recessed by 37 Å. The model suggests that rapamycin–FKBP12 inhibits in part by drastically reducing the accessibility of the already constricted catalytic cleft. This is consistent with the extent of mTORC1 inhibition being
substrate and phosphorylation-site dependent\textsuperscript{25} (additional discussion in Supplementary Fig. 6 legend).

**FRB recruits S6K1 to the active site**

The rapamycin-binding site maps to the FRB surface closest to the active site, in a region of highly conserved residues (Fig. 4a). Four of these residues are invariant in 22 mTOR orthologues from yeast to human, and another four in 21 orthologues, in stark contrast to the FRB surface opposite the active site (Fig. 4a and Supplementary Fig. 7). This raises the possibility that the rapamycin-binding site interacts with substrates to facilitate their entry to the recessed active site.

This model is supported by the observation that mutation of Ser 2035, a rapamycin contact\textsuperscript{26,28} at the centre of the conservation region, reduces phosphorylation of S6K1 and 4E-BP1\textsuperscript{33}. It can also explain inhibition by the rapamycin-binding site interacting with other parts of mTOR, or with associated proteins, especially because rapamycin was thought to be an allosteric inhibitor\textsuperscript{25}. To rule this out, we assayed mTOR\textsuperscript{AN_mLST8}–mLST8 and found that rapamycin, but not the closely related FK506 macrolide, inhibits S6K1\textsuperscript{18} overall phosphorylation, as measured by \textsuperscript{32}P incorporation (Fig. 4b). Inhibition reaches \textasciitilde 45\% at the 20 \textmu M solubility limit of rapamycin, close to the free rapamycin–FRB dissociation constant. Phosphorylation of the key Thr 389 site of the S6K1 hydrophobic motif, measured by a phospho-Thr-389-specific antibody, is inhibited by \textasciitilde 80\% (Fig. 4b).

In a complementary approach, we tested whether the isolated FRB domain inhibits phosphorylation in trans. Figure 4c shows that wild-type FRB but not the Ser2035Ile FRB mutant\textsuperscript{28,30} inhibits overall S6K1\textsuperscript{18} phosphorylation by \textasciitilde 50\% and Thr 389 phosphorylation by \textasciitilde 75\% at the highest concentration tested. To map the region of S6K1 involved in FRB interactions, we reasoned that its deletion should reduce Thr 389 phosphorylation and also render any residual phosphorylation insensitive to rapamycin. By testing successively truncated polypeptides from the S6K1 tail, we found that both criteria are met by residues 393–398 (Fig. 4d). In addition, residues 399–402 and 403–410 probably contribute to FRB interactions, because their truncation progressively reduces phosphorylation (Supplementary Fig. 8). Amino-terminal truncations up to residue 381 have no significant effect.

Together, these data indicate that the FRB provides a secondary substrate-recruitment site near the entrance of the catalytic cleft. We presume that, although the TOS motif, and possibly others, is the primary means of substrate recruitment, the secondary site may facilitate substrate entry into the otherwise restricted active site and may also provide an additional level of specificity, at least for a subset of substrates.

A bipartite substrate-recruitment mechanism is analogous to what has been proposed for the phosphatase calcineurin, the target of
FK506–FKBP12. Most calcineurin substrates require both a primary and a secondary recruitment motif for efficient dephosphorylation, and FK506–FKBP12 inhibits by binding to the secondary recruitment motif site.

**FAT domain**

The FAT domain contains 28 α-helices arranged as α-α-α-helical repeats. Helices α1 to α2 belong to the TPR repeat family and form three discontinuous domains (TRD1, TRD2 and TRD3). Helices α23 to α28 belong to the HEAT family and form a single domain (HRD). The four domains pack sequentially to form a 'C'-shaped α-solenoid that clamps onto the kinase domain (Supplementary Fig. 9a, b). TRD1 interacts with the C-lobe on one side of the kinase domain, and after TRD2 and TRD3 traverse to the other side, the HRD interacts with both the N-lobe and the C-lobe of the kinase domain (Supplementary Fig. 9c, d).

The contacts TRD1 and HRD make to the kinase domain involve conserved residues, consistent with the two interfaces being important for the structure and function of mTOR. For example, three sets of buried hydrogen bond contacts (Glu 1401 to Arg 2317 for TRD1, and Arg 1905 to Glu 2419 and Gln 1941 to Gln 2200 for HRD) involve residues invariant in 22 mTOR orthologues (Supplementary Fig. 9d).

The TRD1 and HRD segments correspond to the FAT segments best conserved among PIKK family members as well, suggesting that the FAT domain clamping onto the kinase domain is a common feature of this family. In addition, PI3Ks contain a HEAT-repeat domain analogous to the HRD in its structure and interactions with other, and to the FAT HRD portion that packs with the kinase domain, although they lack the rest of the FAT domain (Supplementary Fig. 9e).

**mLST8**

mLST8 consists of seven WD40 repeats. At the narrow end of the mLST8 β-propeller structure, a surface that extends across six WD40 repeats binds to both helices and the intervening loop of the LBE helix-loop-helix structure. The interface involves mostly polar LBE residues and polar or aromatic mLST8 residues, and it is dominated by hydrogen bonds (Supplementary Fig. 10).

mLST8 is thought to be a requisite activating subunit of mTOR complexes. The structure suggests that the extended interaction surface of mLST8 may directly stabilize the LBE structure and indirectly influence the organization of the active site through the LBE/FATC/activation-loop spine of interactions (Supplementary Fig. 4a). In support of this, we note that lack of mLST8 results in the association of mTOR with heat shock proteins, and we find that the solubility of overexpressed mLST8 is highly dependent on mLST8 co-expression (not shown). In addition, the growth-suppression phenotype of a temperature-sensitive yeast lsb8 allele can be rescued by mutations either at the LBE hydrophobic core (Ala2290Val) or at the following k25 helix (Leu2302Gln), and the structure is consistent with these mutations stabilizing the local LBE structure.

**Control of mTOR kinase activity**

The proposal that active-site restriction is a negative regulatory mechanism is supported by hyperactivating mutations, most of which map to structural elements involved in limiting active-site access. A large number of mutations cluster at the end of the catalytic cleft that is plugged by k9b (Fig. 5 and Supplementary Fig. 11). They map to the k3, k9, k9b and k10 helices that pack extensively with each other, and to the FAT HRD portion that packs with k3 and k9. These mutations are likely to loosen the k9b-centred structural framework that restricts access from this end of the cleft, presumably without destabilizing the entire active-site structure. The role of the FAT clamp is highlighted by the widely studied Glu2419Lys activating mutation, which eliminates the Glu 2419/Arg 1905 salt bridge at the k9–HRD interface (Supplementary Figs 9c and 11).

Two other hyperactivating mutations map to a portion of the k21 helix that is sandwiched between the FRB base and the N-lobe β-sheet. These mutations (Ile62017Val and Ala2020Val) are unlikely to unfold the FRB, because an FRB hydrophobic core mutation (Trp2027Phe) abolishes kinase activity. The structure suggests that these mutations may loosen the rigid coupling of the FRB to the catalytic cleft, increasing access to the active site.

A third cluster of mutations maps to the N-terminal portion of the FAT domain and to structural residues of TRD1 and the TRD1-proximal portion of TRD2. These mutations would probably destabilize interactions between TRD1 and the kinase domain through loss of structural integrity. They may also destabilize the interactions between HRD and the kinase domain, because the structural coupling of TRD1 and HRD would thermodynamically couple their kinase domain contacts. In addition, the disposition of the N-terminal mTOR segment and its associated RAPTOR could change or become more flexible relative to the kinase domain.

**Inhibitors of the ATP-binding site**

To explore the determinants of inhibitor potency and specificity, we determined the structures of mTORAN–mLST8 bound to Torin2 and PP242, which are highly specific for mTOR, and to P1-103, which has dual specificity for mTOR and class I PI3Ks (Supplementary Table 3).

The overall orientation of Torin2 in the ATP site is as predicted (Fig. 6a and Supplementary Fig. 12a). The tricyclic benzophenanthридinone ring binds to the adenine site and makes a hydrogen bond with the ‘hinge’ between the N and Clobes, analogous to one of two backbone hydrogen bonds made by ATP and diverse inhibitors of PI3 or protein kinases. The aminopyridine group reaches into the ‘inner hydrophobic pocket’, an area at the back of the cleft that many kinase inhibitors...
contact. However, it does not make the three predicted hydrogen bonds \(^4\) (to Asp 2195, Asp 2357 and Tyr 2225). Rather, the structure reveals that a key interaction is the extensive stacking of the tricyclic benzonaphthyridinone ring with the indole group of Trp 2239 from the hinge (Fig. 6a). In the ATP\(_S\) complex, Trp 2239 stacks partly with a three-atom portion of the adenine. In the Torin2 complex, by contrast, Trp 2239 stacks with a ten-atom portion of the tricyclic Torin2 ring. This probably makes a substantial contribution to the subnanomolar potency \(^4\) of Torin2. In addition, because Trp 2239 is not present in canonical protein kinases or in PI3Ks, it would also contribute to the \(\sim 800\)-fold specificity of Torin2 for mTOR over PI3Ks \(^4\). The structure also reveals that the Torin2 trifluoromethyl group packs into an N-lobe pocket (Ile 2163, Pro 2169 and Leu 2185; Fig. 6a). These contacts are less extensive than those made to Trp 2239, and only Leu 2185 is variable across the PIKK and PI3K families. They may contribute to Torin2–mTOR specificity, however, because this portion of the N-lobe \(\beta\)-sheet exhibits considerable variation in its relative orientation in PI3K structures.

PP242 consists of the adenine-mimetic pyrazolopyrimidine scaffold common to PI3 or tyrosine kinase inhibitors, with a hydroxyindole substituent at a position that often points to the inner hydrophobic pocket \(^5\). In mTOR, this pocket is lined with residues conserved across the PI3K and PIKK families, and the selectivity of PP242 for mTOR was unexpected \(^4\). The structure reveals that, although these two PP242 groups are generally positioned as predicted \(^4\), mTOR undergoes a conformational change that expands and deepens the inner hydrophobic pocket, with the PP242 hydroxyindole group reaching deep into the new space (Fig. 6b and Supplementary Fig. 12b). The conformational change involves the Tyr 2225 side chain at the back of the pocket swinging out of the way of the hydroxyindole group (by \(\Delta \gamma_1 = 108^\circ\); Fig. 6c and Supplementary Fig. 12c). Because Tyr 2225 is part of the local hydrophobic core between the N and Clobes, this necessitates additional concerted changes. The Leu 2354 side chain rotates away from the incoming Tyr 2225 (\(\Delta \gamma_1 = 166^\circ\)), the Gln 2223 side chain moves in to plug a resulting gap and a three-residue main-chain atoms within hydrogen-bonding distance. b, PP242–mTOR structure, represented as in a. c, Conformational change in the inner hydrophobic pocket of mTOR on PP242 (cyan) binding. Arrows indicate side-chain rotations and main-chain shifts compared to ATP\(_S\)-bound mTOR (grey). View looking down the vertical axis of b. d, PI-103–mTOR structure, represented as in a.

Conclusions

The mTOR\(^{\text{DN}}\) structure reveals an intrinsically active but otherwise highly restricted catalytic centre, and, together with activating mutations, points to substrate recruitment as a major mechanism controlling the kinase activity. Our biochemical data on S6K1 indicate that in addition to a primary recruitment motif, a secondary recruitment motif proximal to the phosphorylation site is needed for efficient phosphorylation. The FRB acts as a gatekeeper by restricting access to the active site while also granting privileged substrates access through its binding site for the secondary motif. Co-crystal structures of ATP-competitive inhibitors reveal multiple determinants of mTOR specificity, including a conformational change deep inside the catalytic cleft.

METHODS SUMMARY

Details of protein expression, crystallization, structure determination and enzyme assays are described in Methods.
28. Bosotti, R., Isacchi, A. & Sonnhammer, E. L. FAT: a novel domain in PIK-related
25. Choo, A. Y. & Blenis, J. Not all substrates are treated equally: implications for
24. Choi, J., Chen, J., Schreiber, S. L. & Clardy, J. Structure of the FKBP12-rapamycin
19. Schalm, S. S., Fingar, D. C., Sabatini, D. M. & Blenis, J. TOS motif-mediated raptor
18. Ma, X. M. & Blenis, J. Molecular mechanisms of mTOR-mediated translational
21. Oshiro, N.
10. Sancak, Y.
9. Kim, D. H.
8. Chen, E. J. & Kaiser, C. A. LST8 negatively regulates amino acid biosynthesis as a
20. Shiu, Y.-C., Lee, S. C., McEwen, J. M., Hsu, P.-H. & Blenis, J. The FKBP12-rapamycin-binding domain is required for FKBP12-rapamycin-associated protein kinase activity and G1 progression. J. Biol. Chem. 274, 4266–4272 (1999).
14. Long, X., Lin, Y., Ortiz-Vega, S., Yonezawa, K. & Avruch, J. Rheb binds and regulates the mTOR substrates p70 S6 kinase and 4E–BP1 through their TOR binding domain. J. Biol. Chem. 278, 566–571 (2003).
12. Oshiro, N. et al. Rho is an essential regulator of SK6 in controlling cell growth in Drosophila. Nature Cell Biol. 5, 559–566 (2003).
11. Long, X., Lin, Y., Ortiz-Vega, S., Yonezawa, K. & Avruch, J. Rheb binds and regulates the mTOR kinase. Curr. Biol. 15, 702–713 (2005).
10. Sato, T., Nakashima, A., Guo, L. & Tamanoi, F. Specific activation of mTORC1 by Rheb G-protein in vitro involves enhanced recruitment of its substrate protein. J. Biol. Chem. 284, 12783–12791 (2009).
9. Kim, D. H. et al. A new pharmacologic action of CCI-779 involves FKBP12-rapamycin complex (CPP) and 4JT6 (PI-103 complex). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to N.P.P. (pavelin@rskcc.org).
8. Chen, E. Y. & Kaiser, C. A. LST8 negatively regulates amino acid biosynthesis as a component of the TOR pathway. J. Cell Biol. 161, 333–347 (2003).
7. Sarbassov, D. D. et al. A novel binding partner of mTOR defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr. Biol. 14, 1296–1302 (2004).
6. Chen, E. Y. & Kaiser, C. A. LST8 negatively regulates amino acid biosynthesis as a component of the TOR pathway. J. Cell Biol. 161, 333–347 (2003).
5. Kim, D. H. et al. A novel binding partner of mTOR defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr. Biol. 14, 1296–1302 (2004).
4. Choi, J., Chen, J., Schreiber, S. L. & Clardy, J. Structure of the FKBP12-rapamycin
3. Keith, C. T. & Schreiber, S. L. PIK-related kinases: DNA repair, recombination, and cell cycle checkpoints. Science 270, 50–51 (1995).
2. Shaw, R. J. & Cantley, L. C. Ras (3/3) and mTOR signalling controls tumour cell growth. Nature 441, 424–430 (2006).
1. Zoncu, R., Efeyan, A. & Sabatini, D. M. mTOR: from growth signal integration to cancer, diabetes and ageing. Nature Rev. Mol. Cell Biol. 12, 21–35 (2011).
METHODS

Protein expression and purification. The boundary of the crystallized human mTOR\(^{\text{DN}}\) (residues 1376–2549) was identified by screening successive N-terminal truncations of mTOR, produced by transfective transfection in HEK293-F cells, for expression levels, solubility and size exclusion chromatography profile (not shown). For large-scale production of mTOR\(^{\text{SN}}\)–mLST8, we generated a HEK293-F cell line that was stably transfected sequentially by modified pcDNA3.1 vectors encoding Flag-tagged mLST8 and Flag-tagged mTOR\(^{\text{SN}}\). In the absence of mLST8 co-expression, the level of soluble mTOR\(^{\text{SN}}\) was substantially lower, and it co-purified with endogenous mLST8 (not shown). The mTOR\(^{\text{SN}}\)–mLST8, full-length human mTOR–mLST8 and human mTORC1 (mTOR–mLST8–RAPTOR) complexes were similarly produced in HEK293-F cell lines that were stably transfected with the Flag-tagged versions of the proteins. Cells were grown as monolayers in 15-cm plates. They were lysed in 50 mM Tris-Cl, pH 8.0, 100 mM KCl, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% (v/v) glycerol, 2 mM diithiothreitol (DTT) and protease inhibitors using French Press. After centrifugation, the Flag-tagged proteins were affinity purified using anti-Flag M2 agarose beads (Sigma). Following cleavage of the Flag tags by tobacco etch virus (TEV) protease, they were purified by ion exchange (MonoQ) and gel-filtration chromatography. The purified complex was concentrated to 5 mg ml\(^{-1}\) by ultrafiltration in 20 mM Tris-Cl, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5% (v/v) glycerol, 10 mM DTT and 1 mM Pefabloc; pH 8.0, and was stored at −80 °C.

Human S6K1 \(^{\text{DN}}\) protein, which contains the kinase-inactivating K100R mutation, was produced by infecting Five High insect cells with a pFastBac baculovirus expressing the Flag-tagged protein. It was purified by affinity chromatography with anti-Flag M2 agarose beads. The protein was concentrated to 8 mg ml\(^{-1}\) in 50 mM Tris-Cl, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5% (v/v) glycerol, 10 mM DTT and 1 mM Pefabloc; pH 8.0, and was stored at −80 °C.

Human 4E-BP1 was overexpressed in the Escherichia coli strain BL21(DE3) from a modified pGEX4T3 vector, and was purified by glutathione affinity chromatography, cleavage of the GST tag with TEV protease, fractionation on a MonoQ column, removal of free GST by glutathione affinity and gel-filtration (Superdex 75) chromatography. The peak fractions were concentrated to 20 mg ml\(^{-1}\) in 20 mM Tris-Cl, 200 mM NaCl, 5% (v/v) glycerol and 10 mM DTT, pH 8.0.

For large-scale production of mTOR\(^{\text{DN}}\)–mLST8, full-length human mTOR–mLST8 and human mTORC1 (mTOR–mLST8–RAPTOR) complexes were similarly produced in HEK293-F cell lines that were stably transfected sequentially by modified pcDNA3.1 vectors encoding mLST8–PI-103, mLST8–PP242 and mLST8–AMPPNP-Mn\(^2\). The mTOR\(^{\text{DN}}\) residues 1376–1384 at the N terminus, residues 1815–1886 in the FAT domain and residues 2437–2491 between k29b and k210 in the kinase domain are disordered. mLST8 residues 1–7 and 325–326 from the N and C termini are disordered. The Ramachandran plot, calculated by PROCHECK, has 88.5%, 11.0% and 0.5% of the residues in the most favoured, additionally allowed and generously allowed regions, respectively. There are no residues in disallowed regions. The \(R_{	ext{free}}\) test set of the native data contains 1,699 reflections.

In vitro kinase assays. All kinase assays were performed in a buffer of 25 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM MgCl\(_2\), 2 mM DTT and 3% (v/v) glycerol, for 30 min at 30 °C, in a final volume of 15 μl. For a typical reaction, the indicated amount of enzymes, substrates and competitors were incubated together for 10 min on ice in the kinase buffer in a final volume of 13.5 μl. Reactions were started by the addition of 1.5 μl of cold ATP (final concentration, 0.5 mM) including 2 μCi of \(\gamma\text{-}^{32}\text{P}\) ATP (6000 Ci mmol\(^{-1}\), Perkin-Elmer). In assays with rapamycin, all reactions in a titration contained a constant level of 3.3% DMSO. Reactions were stopped by the addition of 15 μl of 2X NuPAGE LDS sample buffer and boiling for 3 min, and were resolved in a 4–12% NuPAGE Bis-Tris gel. Where applicable, each reaction was split into two for analysis by phosphoimaging and immunoblotting. All antibodies were obtained from Cell Signaling Technology, except for the anti-Flag antibody, which was obtained from Sigma.

49. Otwinowski, Z. & Minor, W. Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. 276, 307–326 (1997).

50. Bricogne, G., Vonrhein, C., Flensburg, C., Schlitz, M. & Paciorek, W. Generation, representation and flow of phase information in structure determination; recent developments and in around SHARP 2.0. Acta Crystalogr. D 59, 2023–2030 (2003).

51. Collaborative Computational Project, Number 4. The CCP4 suite: programs for protein crystallography. Acta Crystalogr. D 50, 760–763 (1994).

52. Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgaard, M. Improved methods for building protein models in electron density maps and the location of errors in the models. Acta Crystalogr. A 47, 110–119 (1991).

53. Adams, P. D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystalogr. D 66, 213–221 (2010).