

1 **Title:** Bipartite binding and partial inhibition links DEPTOR and mTOR in a mutually
2 antagonistic embrace

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6

7 **Abstract**

8 mTORC1 is a kinase complex regulating cell growth, proliferation and survival. Because mis-
9 regulation of DEPTOR, an endogenous mTORC1 inhibitor, is associated with some cancers,
10 we reconstituted mTORC1 with DEPTOR to understand its function. We find that DEPTOR
11 is a unique *partial* mTORC1 inhibitor that may have evolved to preserve feedback inhibition
12 of PI3K. Counterintuitively, mTORC1 activated by RHEB or oncogenic mutation is much
13 more potently inhibited by DEPTOR. Although DEPTOR partially inhibits mTORC1,
14 mTORC1 prevents this inhibition by phosphorylating DEPTOR, a mutual antagonism that
15 requires no exogenous factors. Structural analyses of the mTORC1/DEPTOR complex showed
16 DEPTOR's PDZ domain interacting with the mTOR FAT region, and the unstructured linker
17 preceding the PDZ binding to the mTOR FRB domain. Here we show, in contrast to previous
18 cellular studies, that both the PDZ and linker regions are essential for inhibition, and it is likely
19 that interaction with the FRB is crucial to the unique partial inhibition.

20

21 **Introduction**

22 The mammalian/mechanistic target of rapamycin complex 1 (mTORC1) is a large (~1 MDa)
23 multiprotein complex consisting of two copies of three subunits: the evolutionary conserved
24 Serine/Threonine protein kinase mTOR (a member of the phosphoinositide-3-kinase-related

1 kinases superfamily of protein kinases, PIKKs), RAPTOR and mLST8 (Brown et al., 1994;
2 Hara et al., 2002; Kim et al., 2003; Sabatini et al., 1994; Sabers et al., 1995). mTORC1 senses
3 and integrates inputs originating from nutrients, growth factor signalling pathways, oxidative
4 stress, and cellular energy levels (Jewell et al., 2013; Laplante and Sabatini, 2012). The net
5 response of mTORC1 to such stimuli is to promote cell growth, by activating protein
6 translation, ribosome biogenesis, lipid and nucleic acid biosynthesis and inhibiting autophagy
7 (Dunlop and Tee, 2014; Laplante and Sabatini, 2012; Loewith and Hall, 2011; Valvezan and
8 Manning, 2019). The effect of mTORC1 on protein synthesis is primarily due to its
9 phosphorylation of S6 kinase 1 (S6K1) and eIF-4E binding protein 1 (4EBP1) (Gingras et al.,
10 1999; Holz et al., 2005; Holz and Blenis, 2005; Kang et al., 2013). Both substrates interact with
11 the mTORC1 complex through their general TOR signalling (TOS) motif that binds the
12 RAPTOR subunit (Schalm and Blenis, 2002; Schalm et al., 2003; Schalm et al., 2005), as well
13 as by forming a second interaction with the FRB helical insertion within the N-lobe of the
14 mTOR kinase domain (Yang et al., 2017). A more elaborate mode of mTORC1 substrate
15 interaction has been suggested for the transcription factor TFEB, which lacks a TOS motif, but
16 instead binds an active Rag heterodimer that can form a complex with RAPTOR (Martina and
17 Puertollano, 2013; Napolitano et al., 2020). It has been proposed that the substrates'
18 multipartite mode of recognition is crucial for substrate specificity for mTORC1 versus
19 mTORC2 (Baretic and Williams, 2014).

20 In addition to its three core components, mTORC1 associates with various subunits that
21 regulate mTORC1 activity or direct its cellular localisation: nutrients such as amino acids
22 promote the association of RAPTOR with heterodimeric Rag GTPases, which, along with the
23 Ragulator complex, recruit mTORC1 to lysosomal surfaces. There, the small GTPase RHEB
24 (Ras homolog enriched in brain) in its GTP-bound state activates mTORC1 via direct
25 interaction with the mTOR catalytic subunit (Anandapadamanaban et al., 2019; Rogala et al.,

1 2019; Yang et al., 2017). Two endogenous negative regulators of mTORC1 activity have been
2 identified to date: DEPTOR (DEP-domain containing mTOR-interacting protein), which also
3 inhibits mTORC2 (Peterson et al., 2009), and PRAS40 (proline-rich Akt substrate of 40 kDa)
4 (Sancak et al., 2007; Vander Haar et al., 2007; Wang et al., 2007). PRAS40 interacts with
5 RAPTOR via a TOS binding motif in a similar fashion as the substrates 4EBP1 and S6K1,
6 resulting in a specificity for mTORC1 (Yang et al., 2017). PRAS40 inhibition is lost upon its
7 insulin-stimulated Akt/PKB-mediated phosphorylation, which decreases its affinity for
8 mTORC1 (Sancak et al., 2007; Thedieck et al., 2007; Vander Haar et al., 2007).

9 Interactions of DEPTOR with the mTOR complexes are less clear. DEPTOR is a 46
10 kDa protein that consists of three distinct and highly conserved regions (from N- to C-
11 terminus): two tandem DEP domains, an unstructured linker of ~100 residues (named long-
12 linker) and a C-terminal PDZ domain. The long-linker contains multiple serine
13 phosphorylation sites as well as a consensus β TrCP1-binding site, SSGYFS (referred to as the
14 DEPTOR phosphodegron). Previously, it was suggested that DEPTOR inhibits mTOR
15 function *in vivo* via an interaction of its PDZ domain with mTOR FAT domain (Peterson et al.,
16 2009). However, the nature of this interaction remained elusive.

17 Under starvation conditions, DEPTOR binds to mTOR and inhibits its kinase activity,
18 whereas under nutrient replete conditions mTOR-dependent phosphorylation at its degron site
19 marks DEPTOR for degradation (Duan et al., 2011; Gao et al., 2011; Wang et al., 2012a; Zhao
20 et al., 2018). Two models of DEPTOR-specific mTORC1 regulation were proposed: one
21 suggesting that mTORC1 activation is due to DEPTOR displacement by the phospholipase D1
22 (PLD1) product phosphatidic acid (PA) (Yoon et al., 2015) and another linking mTORC1
23 inhibition to deubiquitylation of DEPTOR (Zhao et al., 2018). Recent reports describe
24 DEPTOR as a negative regulator of mTOR function rather than an inhibitor, as residual mTOR
25 activity is observed in the presence of DEPTOR, *in vivo*, dampening but not eliminating S6K1

1 and 4EBP1 phosphorylation (Caron et al., 2016; Dong et al., 2017; Hu et al., 2017; Laplante et
2 al., 2012; Li et al., 2014). As the mTOR pathway is constitutively activated in cancer, levels of
3 DEPTOR are low in most tumours. Exceptions are a subset of multiple myeloma (MM), thyroid
4 carcinoma and lung cancers (Wang et al., 2012b), where overexpression of DEPTOR
5 eliminates feedback inhibition downstream of mTORC1, resulting in hyperactivation of
6 PI3K/mTORC2/Akt signalling and thereby stimulating cell survival (Peterson et al., 2009).
7 RNAi-silencing of overexpressed DEPTOR in a set of MM cells resulted in stalling cell growth
8 and triggering apoptosis, suggesting DEPTOR regulation could be a viable therapeutic strategy
9 (Peterson et al., 2009). Indeed, small molecule inhibitors that were recently reported to prevent
10 the formation of an mTOR/DEPTOR complex showed selective cytotoxicity against MM cells
11 (Lee et al., 2017; Shi et al., 2016; Vega et al., 2019). To assist future inhibitor design, we
12 determined the structural and kinetic basis for DEPTOR regulation of mTORC1. The cryo-EM
13 reconstruction of mTORC1 in complex with DEPTOR at 4.3 Å resolution reveals a bipartite
14 binding mechanism of DEPTOR to mTORC1. Specifically, DEPTOR's long-linker and PDZ
15 domain interact with the FRB and FAT domains of mTOR, respectively, and these interactions
16 were validated with NMR and HDX-MS. Kinetic analysis narrowed down DEPTOR's minimal
17 inhibitory unit to the long-linker-PDZ region, revealing that, in contrast to previous cellular
18 studies, the PDZ domain alone is not sufficient for mTORC1 inhibition, and showed that
19 DEPTOR is a unique partial inhibitor. Remarkably, the DEPTOR/mTORC1 complex
20 possesses kinase activity, so that maximal mTORC1 inhibition by DEPTOR leaves the
21 complex with about ~50% residual activity.

22

23 **Results**

24 **DEPTOR is a partial inhibitor of mTORC1 *in vitro*, independent of substrate identity.**

1 To characterise the mechanism of mTORC1 inhibition by DEPTOR, we carried out
2 reconstituted inhibition assays with purified recombinant mTORC1, DEPTOR and two major
3 mTORC1 substrates, 4EBP1 (wild-type) and S6K1 (GST-tagged S6K1³⁶⁷⁻⁴⁰⁴ polypeptide).
4 Using immunoblotting with antibodies specific for the phosphorylated substrates, we found
5 that DEPTOR inhibited mTORC1 with half maximal inhibitory concentration (IC₅₀) of 14 μM
6 for wild-type 4EBP1, and 51 μM for S6K1³⁶⁷⁻⁴⁰⁴ (Figure 1A). Interestingly, inhibition of
7 mTORC1 by DEPTOR for both substrates appeared to be partial, with mTORC1 having about
8 50% residual activity at even the highest concentrations of DEPTOR that could be achieved.
9 This residual activity could also be detected using Phos-tag gels, that efficiently separate
10 phosphorylated from non-phosphorylated proteins (Figure 1-figure supplement 1A.B). In
11 contrast, the mTORC1 specific inhibitor PRAS40 showed full inhibition under identical
12 conditions (Figure 1B).

13 Partial enzyme inhibitors bind to the enzyme and decrease its activity, but still allow
14 substrate turnover, even with the inhibitor bound (Grant, 2018). Consequently, a partial
15 inhibitor cannot bind in a manner that completely occludes substrate binding. To test whether
16 DEPTOR influences substrate binding, we measured the K_M for 4EBP1 in the presence and
17 absence of DEPTOR (Figure 1C). Previously, two distinct $K_{M,4EBP1}$ have been detected, which
18 represent independent binding at the TOS motif binding site on the RAPTOR subunit ($K_{M1} =$
19 1.8 μM) and at the FRB binding site on the mTOR subunit ($K_{M2} = 585$ μM) (Figure 1A) (Yang
20 et al., 2017). Increasing concentrations of DEPTOR had no apparent effect on the $K_{M,4EBP1}$ of
21 the TOS motif binding site (K_{M1}), suggesting that DEPTOR is not able to compete with 4EBP1
22 at this site (Figure 1C). The affinities for the FRB binding were too low to get a reliable kinetic
23 result. Nevertheless, our structural analyses (described below) imply a very similar binding site
24 at the FRB domain for the DEPTOR long-linker and for the 4EBP1/S6K1 substrates,
25 suggesting a partially competitive binding mechanism for this site. The mTORC1 inhibitor

1 PRAS40, which shares the TOS binding site with 4EBP1, shows full inhibition of 4EBP1
2 phosphorylation under our assay conditions (Figure 1B). DEPTOR's inability to compete at
3 the TOS-motif binding site suggested that inhibition of 4EBP1 phosphorylation may be partial
4 because of unhindered binding of 4EBP1 at the high affinity TOS site located on RAPTOR.
5 To examine this proposition, we employed a TOS-less 4EBP1 mutant as a substrate. DEPTOR
6 seemed to compete more effectively with this mutant, since the IC_{50} was two-fold lower with
7 this modified substrate, however, the residual activity remained at about 50% at the maximum
8 DEPTOR concentration (Figure 1A). As 4EBP1 binding has been reported to involve
9 additional sites, like the RAIP motif (Beugnet et al., 2003; Eguchi et al., 2006), we next used
10 a simpler substrate, consisting of a short S6K1 peptide that is expected to exclusively bind to
11 the FRB site (Yang et al., 2013). Residual activity in the presence of DEPTOR was also
12 observed with this substrate (Figure 1A). Based on these observations, we concluded that
13 partial inhibition by DEPTOR is an intrinsic property of DEPTOR, which takes place
14 independently of substrate identity and substrate binding mode.

15 To test the possibility that the residual activity is caused by half-site reactivity in
16 mTORC1 via allosteric communication across the dimer interface, we tested DEPTOR
17 inhibition of a monomeric form of mTOR (the mTOR^{AN}-mLST8 complex, which lacks the N-
18 terminal 1375 residues of mTOR and the RAPTOR subunit). The IC_{50} for the monomeric
19 mTOR^{AN}-mLST8 was 0.4 μ M, and the residual activity 33% (Figure 1D). Since DEPTOR
20 could still not fully inhibit this monomeric mTOR, we conclude that DEPTOR's residual
21 activity was not caused by effects related to the dimeric nature of mTORC1.

22

23 **The minimal inhibitory unit of DEPTOR is the long-linker-PDZ.**

24 To determine DEPTOR's mechanism of inhibition of mTORC1 in more detail, DEPTOR
25 deletion variants as well as a 13S/T→A mutant with most of the phosphorylation sites in

1 DEPTOR removed (Peterson et al., 2009) were designed to identify the regions that are crucial
2 for inhibition and the role of DEPTOR phosphorylation in the mechanism (Figure 2A). It was
3 reported that DEPTOR's PDZ domain alone (residues 324-409) was sufficient to inhibit mTOR
4 when transiently overexpressed in cells (Peterson et al., 2009). However, our *in vitro* assay
5 showed no measurable inhibition by the PDZ domain alone (Figure 2B and C). Furthermore,
6 there was no inhibition with PDZ preceded by a short section of the linker (short-linker,
7 described below, Figure 2-figure supplement 1A). Instead, a DEPTOR construct that includes
8 the long-linker region preceding the PDZ *in addition to* the PDZ domain comprises the minimal
9 inhibitory unit. Overexpression of the DEPTOR PDZ domain alone may have an indirect effect
10 on mTOR signalling in cells. Recently, an interaction of DEPTOR PDZ with the C-terminal
11 portion of pREX2, a PTEN inhibiting protein that includes domains structurally related to
12 DEPTOR, has been identified (Fine et al., 2009; Yen et al., 2012). Considering the intertwined
13 nature of the PI3K/mTOR pathways with numerous feedback loops and crosstalk modes, it is
14 difficult to unambiguously identify the origin of mTOR inhibition by the PDZ domain of
15 DEPTOR in cell-based experiments. The tandem DEP domains alone (residues 1-221) or in
16 combination with the long-linker (residues 1-323) showed no inhibition (Figure 2B and C).
17 The function of the DEP domains remains therefore unknown.

18 DEPTOR is phosphorylated in an mTOR dependent manner in cells, and
19 phosphorylation sites have been identified in the long-linker region (Duan et al., 2011; Gao et
20 al., 2011; Peterson et al., 2009; Wang et al., 2012a; Zhao et al., 2018). To check the possibility
21 that the inhibition of 4EBP1 and S6K1 phosphorylation by DEPTOR is due solely to DEPTOR
22 acting as an alternative substrate for mTORC1, we tested inhibition of mTORC1 by a
23 previously described DEPTOR mutant in which 13S/T residues in the long-linker region were
24 mutated to alanine (13A mutant) (Peterson et al., 2009). The IC₅₀ of this DEPTOR mutant was
25 comparable to the wild-type DEPTOR for both the wild-type mTORC1 (Figure 2D) and

1 mTORC1 with an activating cancer-associated mTOR mutation (A1459P, Figure 2-figure
2 supplement 1C), so competition with substrate in the active site is not the principal mechanism
3 for DEPTOR inhibition of mTORC1, suggesting that DEPTOR reduces substrate turnover via
4 an allosteric mechanism.

5 We next assayed phosphorylation of full-length DEPTOR and its deletion variants via
6 Phos-tag SDS PAGE. To increase the signal of phosphorylated DEPTOR for reliable detection,
7 we used the hyperactive mTORC1 A1459P. The isolated PDZ as well as tandem DEP domains
8 were not phosphorylated at any detectable rate in an mTOR dependent manner (Figure 2-figure
9 supplement 1D), while the DEPDEP-long-linker was phosphorylated at a reduced rate
10 compared to wild-type DEPTOR at equimolar concentrations (Figure 2-figure supplement 1E).
11 This is an additional indication that the PDZ domain assists the DEPTOR long-linker
12 interaction with mTORC1.

13

14 **Cryo-EM structure of mTORC1/DEPTOR reveals a bipartite binding mode of DEPTOR** 15 **to mTOR.**

16 We determined the structure of mTORC1 bound to full length DEPTOR by electron cryo-
17 microscopy (cryo-EM). Using cross-linked mTORC1/DEPTOR, we generated a 4.3 Å
18 resolution reconstruction of mTORC1 in a complex with DEPTOR (Figure 3A). The overall
19 architecture of the mTORC1 complex bound to DEPTOR resembles the conformation of
20 mTORC1 in the absence of RHEB (PDB 6BCX). However, an additional density can be
21 observed in a crevice between the FAT domain and the N-head of mTOR, centred at residues
22 1527-1571 in the FAT domain. The shape and size of this extra density is consistent with the
23 structure of a PDZ domain (Figure 3C).

24 The density for the DEPTOR PDZ domain was poorly resolved. As crystallization of
25 the 324-409 construct remained unsuccessful, the structure of the DEPTOR PDZ domain was

1 determined using NMR spectroscopy (Figure 3B). Here, instead of calculating a formal
2 solution structure of the PDZ domain, NMR restraint-based models were created using the
3 ROSETTA software suite. The assigned backbone chemical shifts, which are uniquely
4 sensitive to the secondary structure in which each residue resides, were employed to more
5 accurately select fragments of homologous proteins using the POMONA webserver. These
6 fragments then were used to calculate the PDZ model using CS-RosettaCM that combines both
7 chemical shifts (CS) and comparative modeling (CM) (Shen and Bax, 2015). An ensemble of
8 structures was then further refined using a limited set of NOE distance restraints, resulting in a
9 homology model that also satisfies solution state parameters that we observed. This process is
10 advantageous as it creates data driven models significantly faster than traditional NMR
11 structure calculation techniques. Figure 3B represents an ensemble of the 10 lowest energy
12 structures calculated using this methodology, with the lowest energy model used in the final
13 cryo-EM structure refinement. The PDZ domain binds to three consecutive helices (α 16,
14 α 17, α 18, residues 1525-1580), in the middle of the C-shaped FAT domain at a corkscrew
15 junction between two helical solenoids (previously referred to as TRD2 and TRD3 (Yang et
16 al., 2013)) (Figure 3C and D). The key interactions in this surface are formed by E1530, E1531,
17 C1534, M1535, R1538, and Q1562 on the mTORC1 FAT domain, and residues 336-342 and
18 354-362 on the DEPTOR PDZ domain.

19 We identified a second density adjacent to the mTOR FRB domain, covering the
20 lipophilic patch formed by the FRB domain residues Y2038, F2038, Y2105, and F2108 (Figure
21 3C and D). It is likely that this density represents part of the DEPTOR long-linker preceding
22 the PDZ domain, since our kinetic results identified the long-linker-PDZ as the minimal region
23 of DEPTOR required for mTORC1 inhibition. Neither the long-linker nor the PDZ interactions
24 alone are sufficient for the inhibition by DEPTOR (Figure 2C). DEPTOR shows a bipartite
25 binding mode, with one interaction involving the FRB, similar to the mTORC1 substrates

1 S6K1 and 4EBP1. However, the interaction of the DEPTOR PDZ domain with mTORC1's
2 FAT domain is unique. The PDZ binding site is remote from the active site and serves as an
3 anchor moiety (Figure 3C), similarly to the TOS motif of the substrates such as 4EBP1 that
4 anchor to the TOS-binding site on the RAPTOR subunit. We cannot discern any density
5 spanning the two DEPTOR binding sites, and HDX-MS suggests that the long-linker is
6 unstructured (Figure 3-figure supplement 1A, Supplementary File 1). Furthermore, an NMR-
7 based secondary structure analysis of the isolated DEPTOR long-linker chemical shifts
8 revealed no regions of secondary structure (Figure 3-figure supplement 1B). Consequently, it
9 is impossible to say whether DEPTOR spans the two mTOR binding sites on one molecule, on
10 different mTOR subunits in the dimer or a combination of both of these modes (Figure 3-figure
11 supplement 1C).

12 When the structures of free mTORC1 (PDB 6BCX), RHEB bound mTORC1 (PDB
13 6BCU) and mTORC1 bound to DEPTOR are aligned locally on the C-lobe of the kinase
14 domain, it is clear that the ATP-binding sites of the free mTORC1 and DEPTOR-bound
15 mTORC1 are very similar and both are distinct from the RHEB-bound conformation (Figure
16 3-figure supplement 1D). Free mTORC1 is present in a continuum of conformations, ranging
17 from an open conformation to a closed conformation that are both very different than the
18 RHEB-bound, activated conformation (Yang et al., 2017). It may be that the predominant
19 conformation captured by cryo-EM for free mTORC1 corresponds to an inhibited
20 conformation that is only capable of slowly turning over substrate and this same conformation
21 is the one predominately bound to DEPTOR.

22

23 **DEPTOR PDZ domain binds to mTORC1 in a non-canonical manner.**

24 Typically, PDZ domains bind their targets via the C-terminal tail of the target protein binding
25 in the PDZ α B/ β B binding groove (Ernst et al., 2014). The cryo-EM density for the DEPTOR

1 PDZ domain bound to mTOR suggests that binding occurs in a non-canonical fashion. The
2 PDZ domain binds to helices in the mTOR FAT domain (Figure 3D). This unique type of
3 interaction was further examined by an NMR binding experiment using the non-labelled 1
4 MDa mTORC1 as a binding partner for the isolated ^2H , ^{13}C , ^{15}N DEPTOR PDZ domain. Large
5 protein complexes like mTORC1 would generally severely impair NMR studies due to
6 increased linewidth caused by slower tumbling. The same would apply for any labelled
7 interactor that binds tightly to the complex and shows a slow dissociation rate. Nonetheless, in
8 case of a K_d in the range of 0.1 μM to 1 mM and an excess of the interactor in the NMR
9 experiment, the effects of binding can be imprinted onto the dissociated small interactor
10 (Maurer et al., 2004). In this case, binding effects on the small interactor can be observed in
11 the bulk unbound and labelled protein. The DEPTOR PDZ/mTORC1 interaction showed ideal
12 properties for this experiment and DEPTOR PDZ binding to mTORC1 could be successfully
13 detected from the pool of free PDZ domain. Based on the line broadening, DEPTOR PDZ
14 residues 338-342 and 358-362 form the interface with mTORC1 (Figure 4A, Figure 4-figure
15 supplement 1). While the region 338-342 involves the canonical binding residues, the region
16 358-362 is part of the PDZ $\beta\text{C}/\alpha\text{A}$ structural element and therefore outside the canonical $\alpha\text{B}/\beta\text{B}$
17 binding groove. The binding interface on DEPTOR PDZ is surprisingly similar to the binding
18 mode of the third PDZ domain of the scaffold protein inactivation-no-afterpotential D (INAD)
19 to the TRP channel in *Drosophila* photoreceptors (Figure 4B), indicating that although this is
20 a rare mode of interaction, it is not unprecedented (Ye et al., 2016).

21

22 **A set of linker regions bind to mTOR's FRB domain.**

23 Because our cryo-EM structure showed a bipartite binding that included the FRB domain, and
24 because the PDZ domain alone is insufficient for mTORC1 inhibition, we attempted to define
25 the region of DEPTOR interacting with mTOR's FRB. A small stretch of extra cryo-EM

1 density in mTOR's FRB domain suggested an interaction of DEPTOR with this mTOR domain
2 (Figure 3C). As it has been previously reported that DEPTOR's long-linker is phosphorylated
3 in an mTOR dependent manner (Peterson et al., 2009), we proposed that a region in the
4 DEPTOR long-linker binds to the FRB. To verify this interaction, binding of the long-linker-
5 PDZ (residues 228-409) to the isolated FRB domain was characterised by NMR. The chemical
6 shift perturbation in the ^1H - ^{15}N BEST-TROSY NMR experiments comparing the bound FRB
7 vs. free FRB confirmed that the long-linker interacts with the FRB domain (Figure 5A, Figure
8 5-figure supplement 1A-C). The two most prominent patches affected by this interaction are
9 located at the FRB residues 2035-2042 in $\text{kf}\alpha 1$ and 2103-2109 in $\text{kf}\alpha 4$, which is consistent
10 with the cryo-EM density (Figure 5A). This suggests a similar binding location of the DEPTOR
11 linker at the FRB as the substrates S6K1 or 4EBP1 (Yang et al., 2017). Similar to the substrates,
12 binding affinity of the DEPTOR long-linker to the FRB is weak, with a K_d estimated by NMR
13 to be $\sim 500 \mu\text{M}$.

14 To map the regions in DEPTOR interacting with the FRB domain, we carried out NMR
15 experiments with the isolated DEPTOR long-linker and HDX-MS experiments with the full-
16 length DEPTOR. The chemical shift perturbation of the DEPTOR long-linker (residues 228-
17 323) bound to FRB vs. DEPTOR long-linker alone in ^1H - ^{15}N BEST-TROSY NMR
18 experiments revealed four patches, each of five residues in the long-linker that were altered by
19 FRB binding, residues 228-232, 244-249, 261-264, and 280-285 (Figure 5B). Indeed, the
20 regions of increased protection observed in full-length DEPTOR in the presence of the FRB in
21 an HDX-MS experiment were in good agreement with the NMR result, as DEPTOR residues
22 230-248 and 275-302 were protected from solvent exchange in the presence of the FRB (Figure
23 5C, Supplementary File 2). Notably, both experiments suggested that there was not one unique
24 FRB-binding motif in the long-linker, but multiple regions capable of interacting. This is not
25 surprising, as it has previously been shown for the mTORC1 substrate 4EBP1 that the FRB-

1 interacting region varies based on the substrate's individual phosphorylation sites (Yang et al.,
2 2017). This most likely also applies to DEPTOR, and many mTOR dependent phosphorylation
3 sites have previously been reported in DEPTOR's long-linker (Duan et al., 2011; Gao et al.,
4 2011; Peterson et al., 2009).

5 All FRB interacting areas in DEPTOR are within 45 residues of the PDZ domain, which
6 could cover a distance of about 160 Å, if in an extended conformation. As the linker showed
7 no indication of secondary structure elements, this length should be sufficient for a bipartite
8 mTORC1 binding interaction involving the DEPTOR PDZ and the long-linker binding either
9 a single mTOR subunit or across the dimer interface (Figure 3-figure supplement 1C).

10

11 **DEPTOR PDZ forms an interaction with the long-linker that controls domain stability**
12 **and creates a unique surface on PDZ.**

13 Secondary chemical shift analysis of the isolated DEPTOR long-linker suggested that the long-
14 linker has no residual secondary structure (Figure 3-figure supplement 1B). However, the
15 linker unexpectedly contributes significantly to the stability of the PDZ domain by interacting
16 along the PDZ surface, following a patch of mostly uncharged and hydrophobic residues. This
17 interaction could be detected due to a chemical shift perturbation for some PDZ domain
18 residues in the ^1H - ^{15}N BEST-TROSY spectrum for a construct consisting of the long-linker
19 PDZ when compared to the ^1H - ^{15}N BEST-TROSY spectrum of the PDZ domain alone (Figure
20 5D, Figure 5-figure supplement 2A). By N-terminal deletion analysis of the long-linker-PDZ,
21 we identified a small portion of the linker that is sufficient to stabilize the PDZ domain: a
22 construct consisting of residues 305-409 (here referred to as short-linker PDZ) showed an
23 increased melting temperature of by about 10 °C (Figure 5E). Furthermore, NMR ^{15}N backbone
24 relaxation experiments including T_1 , T_2 and $^{15}\text{N}\{^1\text{H}\}$ heteronuclear NOE analyses showed that
25 the PDZ alone (PDZ-only, residues 324-409) has a high flexibility in the N-terminal region,

1 encompassing the first β strand and the subsequent loop (Figure 5-figure supplement 2B). This
2 suggests that the short-linker region significantly adds to the overall stability of the PDZ
3 domain, but the short-linker-PDZ is not an mTORC1 inhibitor (Figure 2-figure supplement
4 1A).

6 **DEPTOR inhibits activated mTORC1 more strongly than basal mTORC1.**

7 Our structural analysis showed that DEPTOR PDZ binds close to a region on the mTOR FAT
8 domain that undergoes a major conformational change induced by the RHEB-GTP binding
9 (Yang et al., 2017). This suggested that DEPTOR inhibition could be altered as a result of
10 activation. To determine the effect of DEPTOR on activated mTORC1 in our reconstituted
11 system, we tested DEPTOR inhibition of RHEB-GTP- or mutation-activated mTORC1.
12 Surprisingly, the IC_{50} for DEPTOR inhibition decreased about 400-fold in the presence of
13 RHEB-GTP compared to mTORC1 alone (Figure 6A), suggesting tighter binding of DEPTOR
14 to the activated mTORC1. A similar reduction in DEPTOR IC_{50} was observed for the cancer-
15 associated mTOR mutant A1459P (Figure 6B). In contrast, the EC_{50} for RHEB-GTP activating
16 wild-type mTORC1 was not significantly affected by the presence of DEPTOR (Figure 6-
17 figure supplement 1A). Cancer-associated mutations clustering around C1483 were shown to
18 involve structure-stabilizing residues (Yang et al., 2017). These mutations significantly lower
19 the activation energy for the transition from ground state to the RHEB-GTP-bound activated
20 state (Yang et al., 2017). The cancer-associated mutant A1459P is in the middle of a helix α_{12}
21 at the major intra-FAT hinge, and our cryo-EM structure of the A1459P mutant bound to
22 DEPTOR (global resolution 4.7Å) shows this helix becomes disordered (residues 1457-1470)
23 (Figure 6C). This disorder likely increases plasticity of the intra-FAT hinge, thereby improving
24 DEPTOR binding. The structure of mutant mTORC1 bound to DEPTOR reveals a widening
25 of the cleft between the mTOR N-head and the PDZ-bound FAT domain, relative to this cleft

1 in the structure of DEPTOR bound to wild-type mTORC1 (Figure 6D). This cleft widening
2 might enable easier access for DEPTOR PDZ to bind to the FAT domain. Consistent with this
3 proposal, we observe fast on/off kinetics for PDZ binding to the mTORC1 mutant A1459P
4 with a K_d of 0.6 μ M determined by SPR (Figure 6E). For the wild-type mTORC1, the K_d is
5 increased ten-fold ($K_d = 7 \mu$ M), and we observe slow on/off kinetics, which suggests that there
6 is a rate-limiting conformational change within wild-type mTOR to facilitate binding of PDZ.
7 Furthermore, a multibody refinement of the mTORC1/DEPTOR cryo-EM data set indicates
8 that one of the major motions in the wild-type mTORC1/ DEPTOR complex lies in the N-heat
9 domain (Figure 6-figure supplement 1B). The proposition that the N-heat in wild-type mTOR
10 restricts DEPTOR PDZ binding is further supported by the 40-fold lowered IC_{50} for DEPTOR
11 inhibition of the mTOR^{AN}-mLST8 (Figure 1D), which does not possess the N-heat domain.
12 Structurally, the interaction of DEPTOR PDZ with the FAT domain does not seem to be
13 significantly altered between mutant and wild-type mTORC1. Despite tight binding of
14 DEPTOR PDZ to the A1459P mutant, there is no inhibition of the mutant by the isolated PDZ
15 domain (without the long linker), even at saturating concentrations (Figure 6-figure supplement
16 1C). This underlines the need for a bipartite interaction for effective mTORC1 inhibition by
17 DEPTOR.

18

19 **Phosphorylated DEPTOR does not inhibit mTORC1.**

20 In order to reconcile our kinetic and binding results suggesting that a cancer-associated,
21 activated mTOR mutant shows increased DEPTOR association, with previous results showing
22 a *decrease* in the amount of DEPTOR co-immunoprecipitated with exogenously expressed
23 mutant mTOR, we examined the effect of sustained mTORC1 activity on the association of
24 DEPTOR with mTORC1. In our kinetic analysis, we used initial rates of mTORC1 before
25 significant substrate depletion. However, under these conditions, there is also very little

1 phosphorylated DEPTOR produced. To investigate whether mTOR-dependent
2 phosphorylation of DEPTOR alters DEPTOR inhibition, we extensively phosphorylated
3 DEPTOR with the activated mTORC1 mutant A1459P and assayed mTORC1 inhibition by
4 this pre-phosphorylated DEPTOR. We found almost no inhibition of mTORC1 with pre-
5 phosphorylated DEPTOR (Figure 7A). This is consistent with results in cells showing that
6 sustained serum stimulation results in less epitope-tagged, overexpressed wild-type DEPTOR
7 being co-immunoprecipitated with RAPTOR compared with the 13 S/T→A mutant (Peterson
8 et al., 2009). Although previous results also have shown that DEPTOR phosphorylation leads
9 to increased ubiquitylation by the F box protein β TrCP and subsequent degradation (Duan et
10 al., 2011; Gao et al., 2011), our reconstituted system with no degradation machinery, formally
11 establishes that phosphorylation of DEPTOR by mTORC1 results in decreased association of
12 DEPTOR with mTORC1. This supports the previously-proposed mechanism of mTORC1 self-
13 regulation of its inhibition (Figure 7B) (Peterson et al., 2009). It suggests that the degradation
14 that is observed in cells happens subsequent to loss of inhibition of mTORC1 by DEPTOR.

15

16 **Discussion**

17 Our structural and functional analysis of reconstituted mTORC1 inhibition by DEPTOR
18 revealed a unique bipartite binding mechanism, involving an interaction of the DEPTOR PDZ
19 with the FAT domain of mTOR and the DEPTOR long-linker interaction with the FRB. We
20 further identified the long-linker-PDZ as a minimal inhibitory unit.

21 Remarkably, DEPTOR only partially inhibits mTORC1 phosphorylation of its two
22 major substrates 4EBP1 and S6K1, independent of the mTOR activation state, while PRAS40
23 fully inhibits mTOR activity under the identical assay conditions. Partial inhibition of
24 mTORC1 by DEPTOR in cells has been noted (reviewed in (Caron et al., 2018)). Several
25 previous reports have suggested that DEPTOR dampens mTORC1 activation, rather than fully

1 inhibiting it, leaving a residual mTORC1 specific phosphorylation of S6K1 (Caron et al., 2016;
2 Dong et al., 2017; Hu et al., 2017; Laplante et al., 2012; Li et al., 2014). However, it has not
3 been established previously that this is due to DEPTOR's unique property of forming an
4 inhibitory complex with mTORC1 that is capable of turning over substrate. This residual
5 mTORC1 activity in the presence of DEPTOR explains why cells inhibited by rapamycin or
6 by DEPTOR overexpression show different phenotypes and why deletion of DEPTOR has a
7 lesser activating effect on mTORC1 in cells than the loss of TSC1/2 (Caron et al., 2018). Our
8 results show that even for purified recombinant components, partial inhibition is an intrinsic
9 property of DEPTOR and does not require additional cellular components.

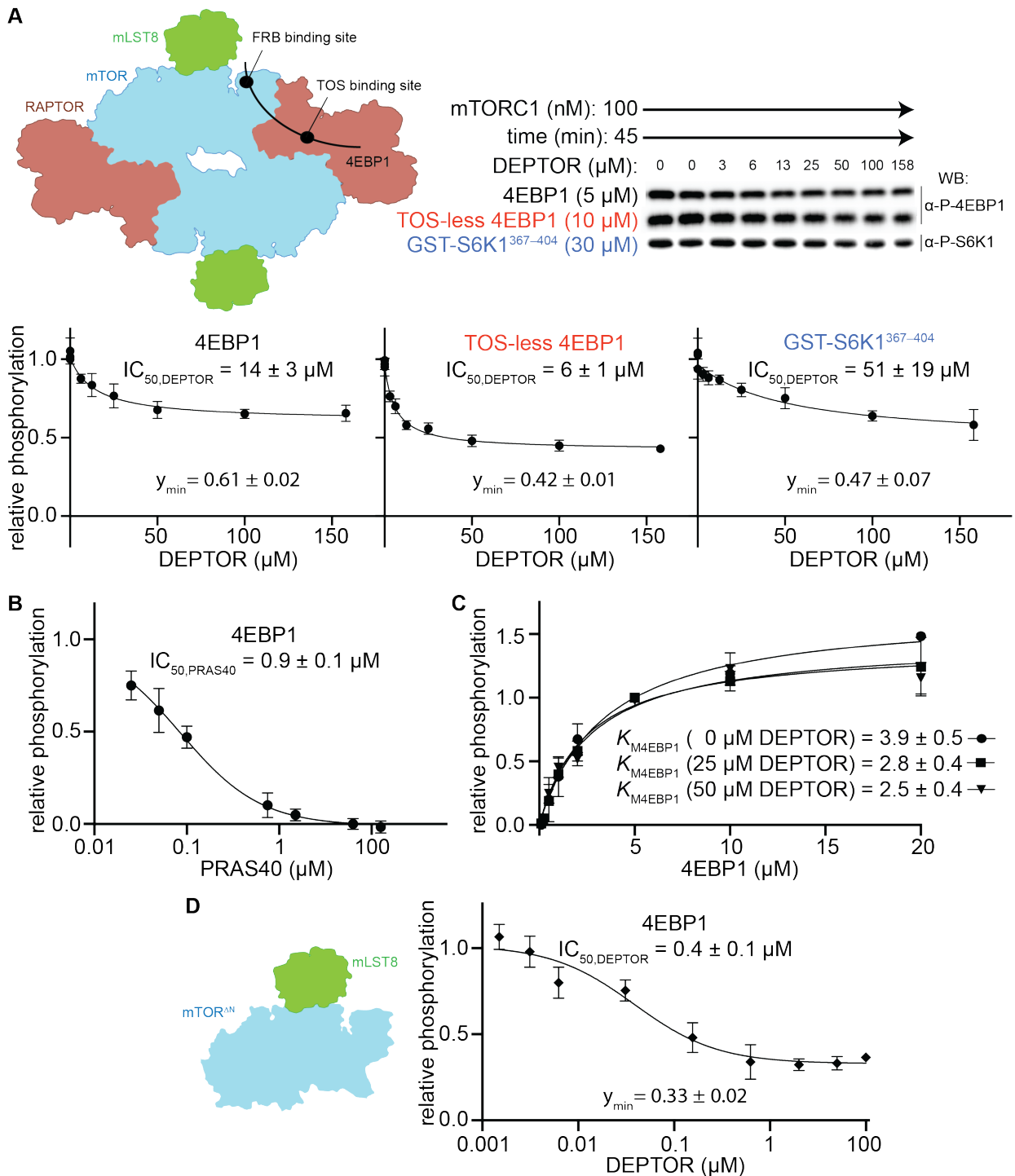
10 The two distinct bipartite binding mechanisms of DEPTOR vs. PRAS40 and the
11 substrates 4EBP1/S6K1 allow a more finely-tuned regulation than single binding sites could
12 provide. DEPTOR, which shares only one binding site with the substrates, allows for residual
13 mTOR activity, while PRAS40, which shares both binding sites with the substrates, results in
14 full enzyme inhibition. An additional layer of control is that the partial inhibition arising from
15 the allosteric inhibition by DEPTOR establishes an upper limit on mTORC1 inhibition. This
16 partial inhibition could be key for keeping the balance in the mTOR/PI3K signalling cascade
17 and mimicking this aspect of DEPTOR might be an objective for design and selection of small-
18 molecule inhibitors of mTORC1. Given that the PDZ domain alone is not sufficient for
19 mTORC1 inhibition, it is likely that the unique partial inhibition that we have demonstrated for
20 DEPTOR is due to the linker interacting with the FRB, and the role of the PDZ interaction with
21 mTOR is to increase affinity of the DEPTOR for mTORC1. A major challenge in inhibiting
22 the PI3K/mTOR pathway is the presence of numerous feedback loops that prevent therapeutic
23 success and give rise to resistance against treatment (reviewed in (Yang et al., 2019)). The use
24 of mTOR inhibitors ultimately results in an overactivation of the PI3K/mTOR pathway due to
25 accumulation of IRS1 and IGF-1R triggering a positive feedback loop which diminishes the

1 therapeutic value of treatment (Britschgi et al., 2012; Dibble et al., 2009; O'Reilly et al., 2006).
2 Downregulating mTORC1 activity while maintaining negative feedback loops could be of
3 therapeutic benefit. A more detailed understanding of mTOR function and ways of partially
4 inhibiting some of the mTOR-dependent processes, while maintaining others might therefore
5 be the key to render mTOR a suitable cancer drug target. DEPTOR is highly overexpressed in
6 Multiple Myeloma cells, which protects these cells from apoptosis (Peterson et al., 2009).
7 Recently, small molecule inhibitors have been designed to interrupt the DEPTOR PDZ/mTOR
8 interaction and have shown selective cytotoxicity against this type of cancer (Lee et al., 2017;
9 Shi et al., 2016; Vega et al., 2019). Efforts like this could significantly benefit from the
10 structural details of the unique non-canonical binding mode of mTORC1 and DEPTOR PDZ
11 described in this study. The new structural insights are important steppingstones towards
12 selectively targeting the mTOR/DEPTOR interaction by pharmacotherapy. In addition to the
13 unique interface formed by mTOR/DEPTOR PDZ interaction, the short-linker/PDZ interface
14 could serve as a second interface to be targeted by small molecule inhibitors, as this interaction
15 has been uniquely observed in DEPTOR PDZ and stability of the PDZ domain is severely
16 impaired when this interaction is lost. The unique features of the mTORC1/PDZ interaction
17 described in this work could be essential for selectively targeting the PDZ domain, which is
18 one of the most common scaffolding domains in the human proteome with about 270 existing
19 versions present in 150 proteins (Harris and Lim, 2001).

20 The discrepancy between the previously-reported observation in cells of reduced
21 DEPTOR binding to activated cancer-associated mutants (Grabiner et al., 2014) and our
22 observation of a lowered DEPTOR IC₅₀ and increased affinity in our reconstituted system for
23 activated mTORC1 might be explained by upregulated cellular processes in cancer cells. One
24 key observation upon DEPTOR discovery was that DEPTOR inhibition quickly vanished after
25 mTORC1 activation, several hours ahead of DEPTOR degradation (Duan et al., 2011; Gao et

1 al., 2011; Peterson et al., 2009). Besides a reduction in DEPTOR expression levels and an
2 increase in its degradation, there might be other factors that drive DEPTOR dissociation upon
3 mTORC1 activation, such as a change in localization of proteins (mTORC1 translocates to
4 lysosomes upon mitogen stimulation (Bar-Peled and Sabatini, 2014)), mTORC1 interactors
5 that could outcompete DEPTOR binding (Yoon et al., 2015), or a change in the DEPTOR
6 phosphorylation levels (Duan et al., 2011; Gao et al., 2011; Peterson et al., 2009), which could
7 decrease mTORC1 affinity and/or increase DEPTOR affinity for other proteins. Grabiner et al.
8 did not compare DEPTOR binding to mitogen stimulated wild-type mTOR with DEPTOR
9 binding to the hyperactive cancer-associated mTOR mutants (Grabiner et al., 2014). We have
10 shown that a cancer-associated, activating mutation alone appears to increase DEPTOR
11 binding, and we found that phosphorylated DEPTOR no longer inhibits mTORC1. These
12 findings suggest that DEPTOR regulation is performed by mTORC1 itself (Figure 7B). In light
13 of mTORC1 remaining partially active in the presence of DEPTOR, it is clear that the kinase
14 itself can release inhibition by DEPTOR. Our results show that an activated mTORC1 binds
15 tighter to DEPTOR, once it phosphorylates DEPTOR, it releases DEPTOR. In contrast,
16 PRAS40 regulation arises from phosphorylation by Akt. Nevertheless, despite the differences
17 in regulation, in both DEPTOR and PRAS40, the negative feedback loop via p-S6K1 onto the
18 PI3K pathway introduces a balance to prevent either too much or too little inhibition by these
19 mTORC1 regulators.
20

1 Figures and legends



2

3 **Figure 1. DEPTOR is a partial inhibitor of mTORC1, independent of substrate identity.**

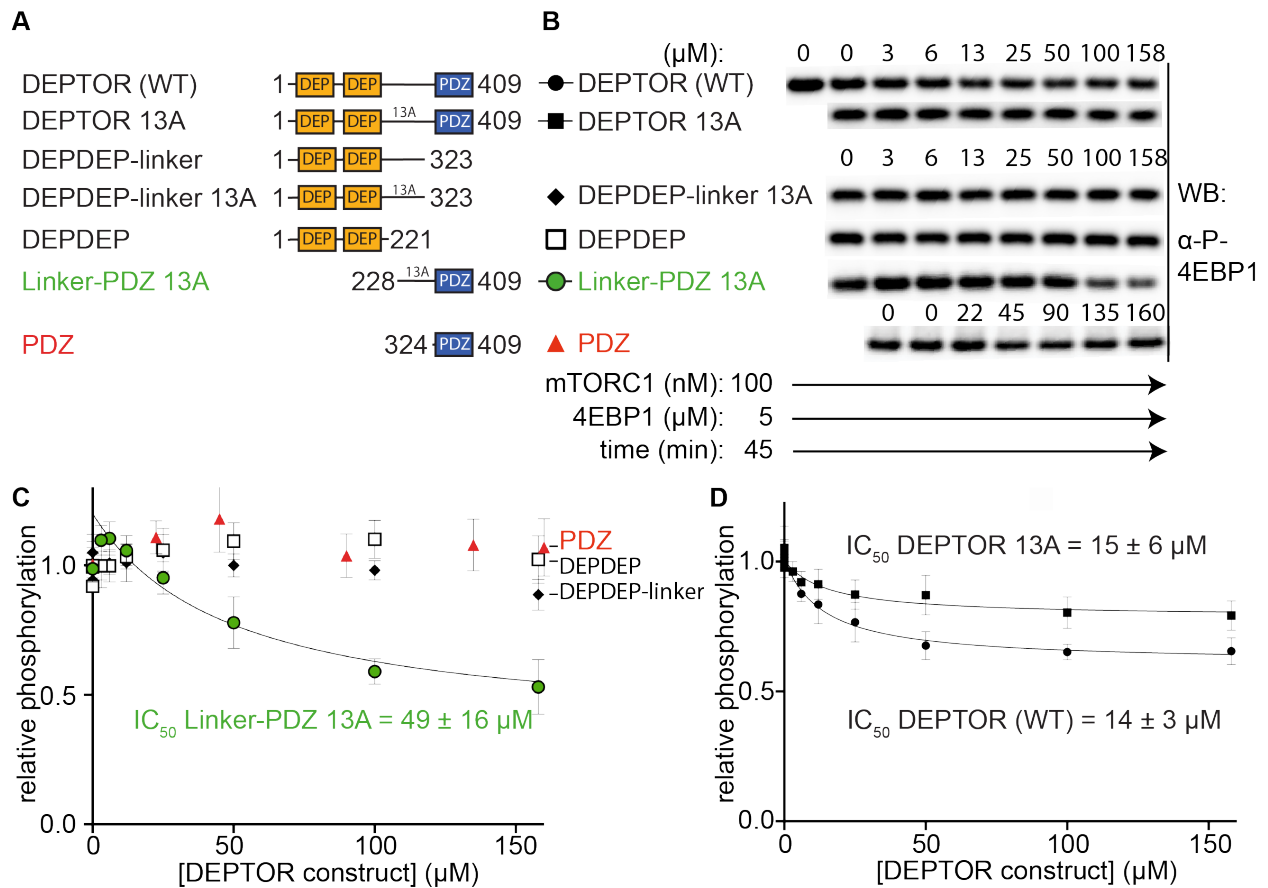
4 (A) Inhibition of the mTORC1 kinase activity by DEPTOR. mTORC1 schematic illustrating
 5 the two known substrate binding sites, the TOS binding site on RAPTOR and the FRB binding
 6 site on mTOR, for 4EBP1 and S6K1 (left panel). Phosphorylation of substrates was analysed

1 by western blots using anti p-4EBP1 (Thr37/46) or anti p-S6K1 (Thr389) primary antibodies
2 (right panel). The S6K1³⁶⁷⁻⁴⁰⁴ peptide encompasses only the FRB-binding site of S6K1.
3 The bottom panels show the quantification of the phosphorylation levels of the substrates based
4 on the western blots from 3 independent experiments (mean \pm SD). Band intensities were
5 normalized to the control (0 μ M DEPTOR) and data were plotted and fit by non-linear
6 regression to determine IC₅₀ and y_{min} (the residual activity at high [DEPTOR]) as described in
7 methods. See Figure 1-figure supplement 1A,B for complementary experiment using Phos-tag
8 SDS PAGE detection.

9 **(B)** Inhibition of mTORC1 by PRAS40. Inhibition of 4EBP1 phosphorylation is complete
10 under identical reaction conditions as carried out for DEPTOR.

11 **(C)** DEPTOR has no effect on the apparent $K_{M,4EBP1}$. The phosphorylation of 4EBP1 in the
12 absence and presence of DEPTOR (25 μ M or 50 μ M), normalized to the 5 μ M 4EBP1 is plotted
13 (mean \pm SD, n \geq 3) and K_M values were fit as described in methods.

14 **(D)** Inhibition of monomeric mTOR^{AN}-mLST8 (left panel) by DEPTOR (mean \pm SD, n \geq 3).
15 Similar to the wild-type mTORC1 complex, partial inhibition is observed (right panel).



1

2 **Figure 2. The minimal inhibitory unit of DEPTOR is long-linker-PDZ.**

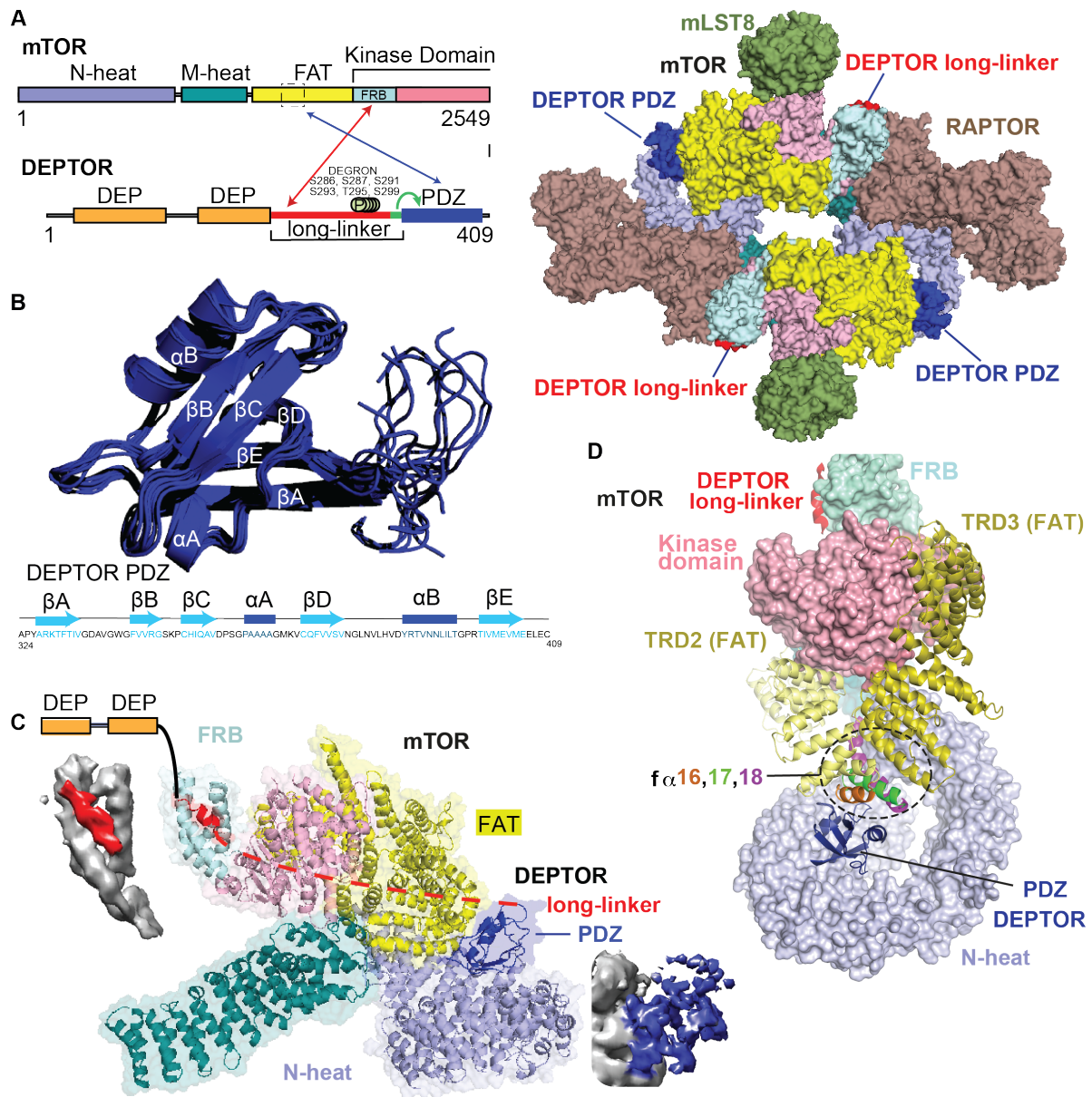
3 (A) DEPTOR deletion variants tested as inhibitors and substrates for mTORC1.

4 (B) Immunoblots showing the residual phosphorylation of 4EBP1 in the presence of various
5 DEPTOR deletion variants.

6 (C) and (D) Quantification of western blots of phosphorylated 4EBP1 plotted as a fraction of
7 the control (0 μM inhibitor) vs. inhibited (mean \pm SD, $n \geq 3$) and fit to a non-linear regression
8 for all deletion variants to determine IC₅₀. While all experiments were performed at 30 °C,
9 inhibition of mTORC1 by the PDZ domain was tested at 20 °C for 20 min as the domain
10 stability was low. N-terminally extended PDZ constructs that showed increased temperature
11 stability showed no inhibition of mTORC1 (Figure 2-figure supplement 1A). To demonstrate
12 that the temperature difference had no effect on the inhibition, DEPTOR (WT) was tested at
13 20 °C for mTORC1 inhibition (Figure 2-figure supplement 1B). The data shown for DEPTOR

1 (WT) is also part of Figure 1A. DEPTOR 13A inhibition of the mTORC1 A1459P mutant is
2 shown in Figure 2-figure supplement 1C.

3



1

2 **Figure 3. Cryo-EM structure of mTORC1/DEPTOR reveals bipartite binding mode of**
 3 **DEPTOR linker-PDZ to mTOR.**

4 (A) Domain organization of mTOR and DEPTOR is shown on the left. The region of
 5 interaction between the mTOR FAT domain residues 1527-1571 and the DEPTOR PDZ
 6 domain, as well as the mTOR FRB domain and the DEPTOR linker are highlighted with
 7 arrows. Surface representation of the model for the mTORC1/DEPTOR complex is shown on
 8 the right, colour-coded by domains. Only the linker and PDZ domain, not the tandem DEP

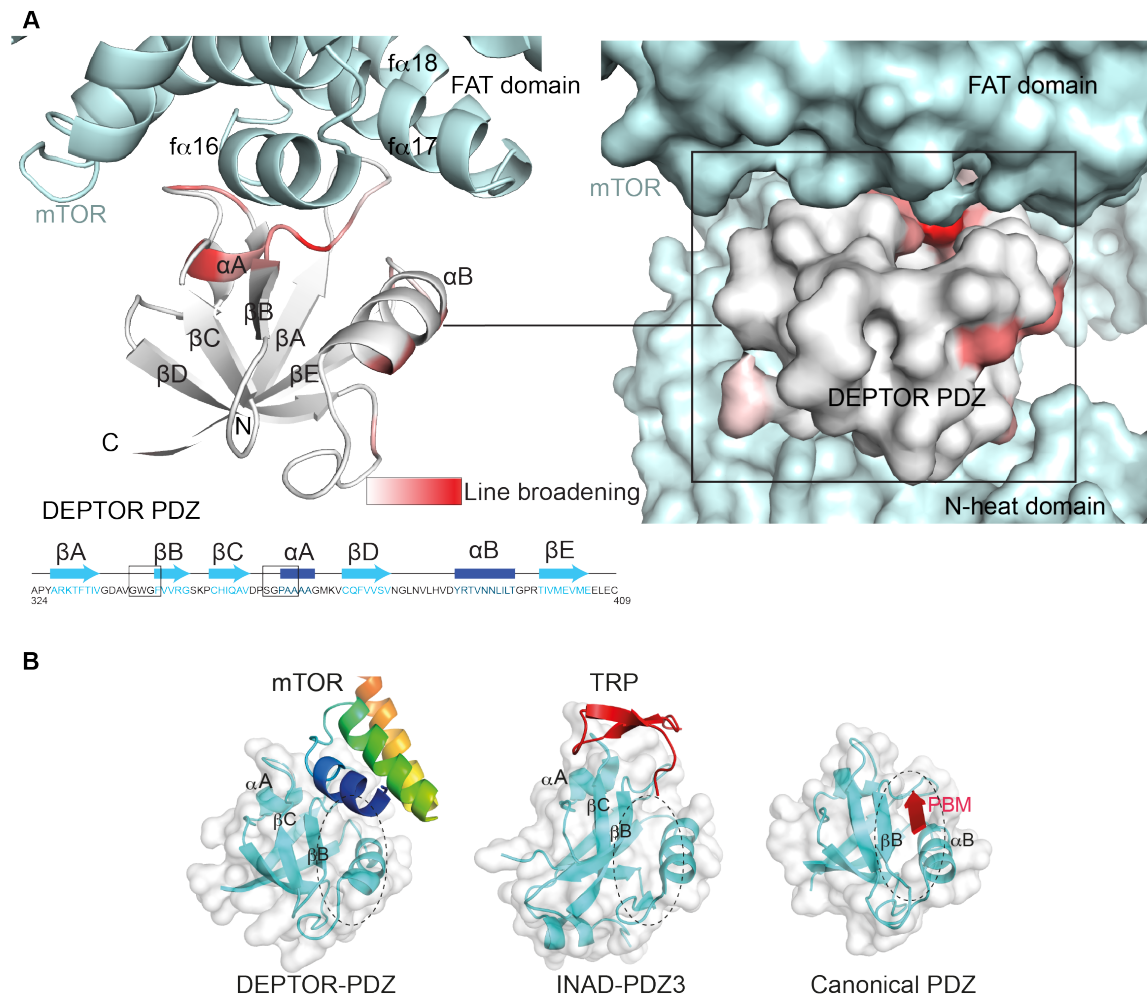
1 domain of DEPTOR are visible in the cryo-EM density. See Figure 3-figure supplement 2 and
2 Table 1 for cryo-EM data details.

3 **(B)** The 10 lowest energy homology models of DEPTOR PDZ produced using CS-Rosetta
4 guided by NMR data, including 14 NOE distance restraints. DEPTOR PDZ construct (324-
5 409) was used. See Figure 5-figure supplement 2A for the assigned ^1H - ^{15}N BEST-TROSY
6 spectrum of the PDZ domain.

7 **(C)** A close-up of DEPTOR binding to the mTOR subunit shows DEPTOR PDZ domain in a
8 crevice between the FAT domain and the N-head of mTOR. DEPTOR long-linker (red) forms
9 interactions at the FRB domain. The dashed line spans the distance of DEPTOR long-linker
10 between the two binding sites. Cryo-EM density for DEPTOR and its binding sites is shown
11 next to the model. The density for mTOR, PDZ and long-linker are coloured in grey, blue, and
12 red, respectively.

13 **(D)** Three mTOR helices ($\alpha 16$, $\alpha 17$, $\alpha 18$) at the junction of two solenoids (TRD2 and
14 TRD3) in the FAT domain are splayed and form a non-canonical interface with the DEPTOR
15 PDZ domain.

16



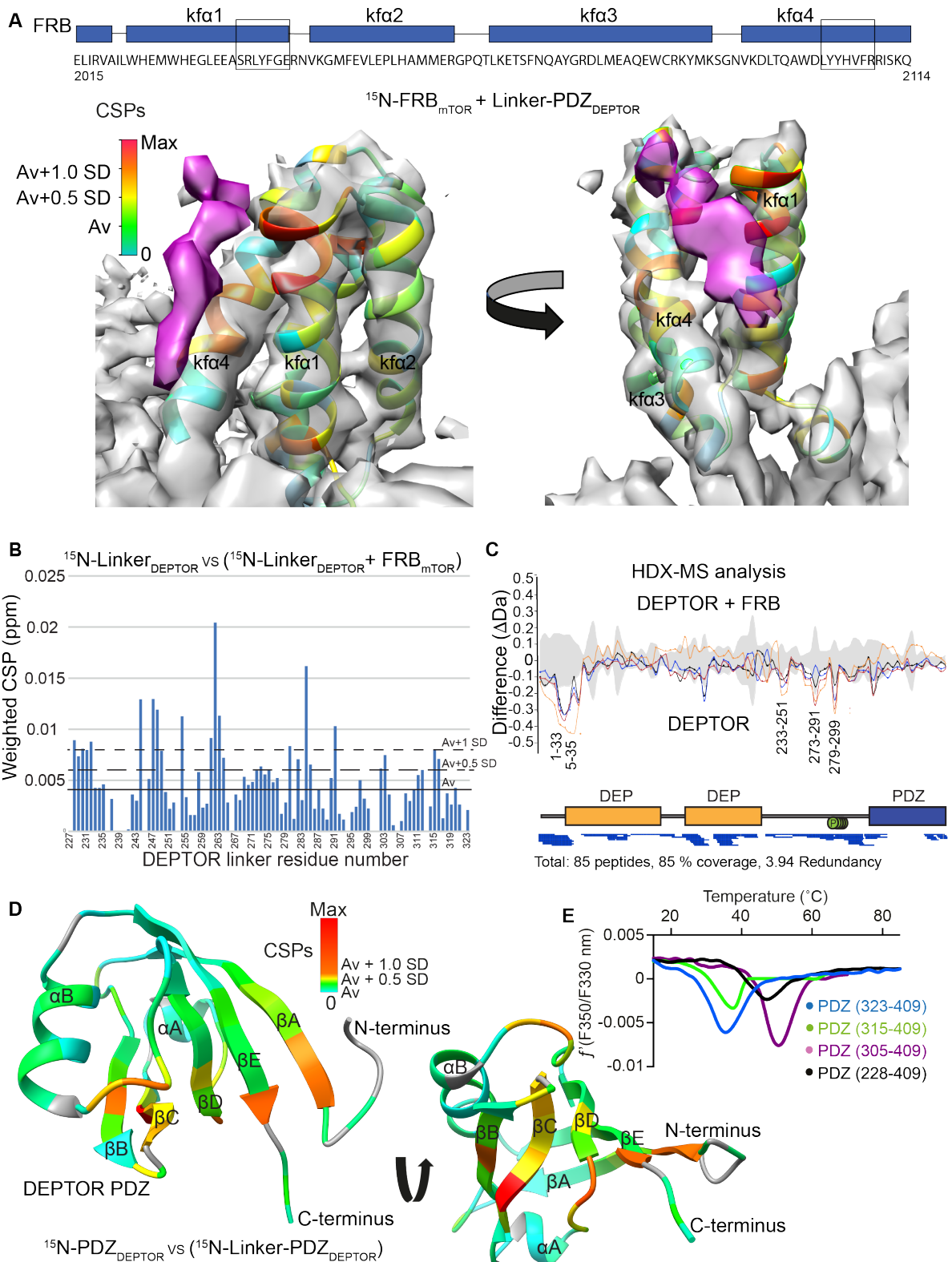
1

2 **Figure 4. DEPTOR PDZ domain binds to mTORC1 in a non-canonical manner.**

3 (A) Line broadening of isotope-labelled DEPTOR PDZ (residues 324-409) caused by its
 4 binding to the full length mTORC1 is displayed in shades of red on the ribbon diagram of the
 5 PDZ domain. The NMR data is consistent with the non-canonical binding mode of DEPTOR
 6 PDZ seen with the cryo-EM. See Figure 4-figure supplement 1 for NMR differential line-
 7 broadening analysis.

8 (B) A comparison of canonical and non-canonical binding to PDZ domains. The canonical
 9 binding mode (PDB 1BE9) is illustrated by the third PDZ domain of postsynaptic density-95
 10 (PSD-95) interacting with the cysteine-rich interactor of PDZ3 (CRIPT). The DEPTOR PDZ
 11 binds to the mTOR FAT region in a non-canonical manner. Distinct non-canonical binding is

- 1 also seen for other PDZ domains, such as the interaction of PDZ3 of inactivation no
- 2 afterpotential D (INAD) with the transient receptor potential (TRP) channel (PDB 5F67).
- 3



1

2 **Figure 5. DEPTOR linker has two parts, each with a distinct role.**

3 (A) Mapping the mTOR FRB surface that binds to the DEPTOR linker by the chemical shift
4 perturbations (CSPs) of the FRB (residues 2015-2114) bound to DEPTOR linker-PDZ

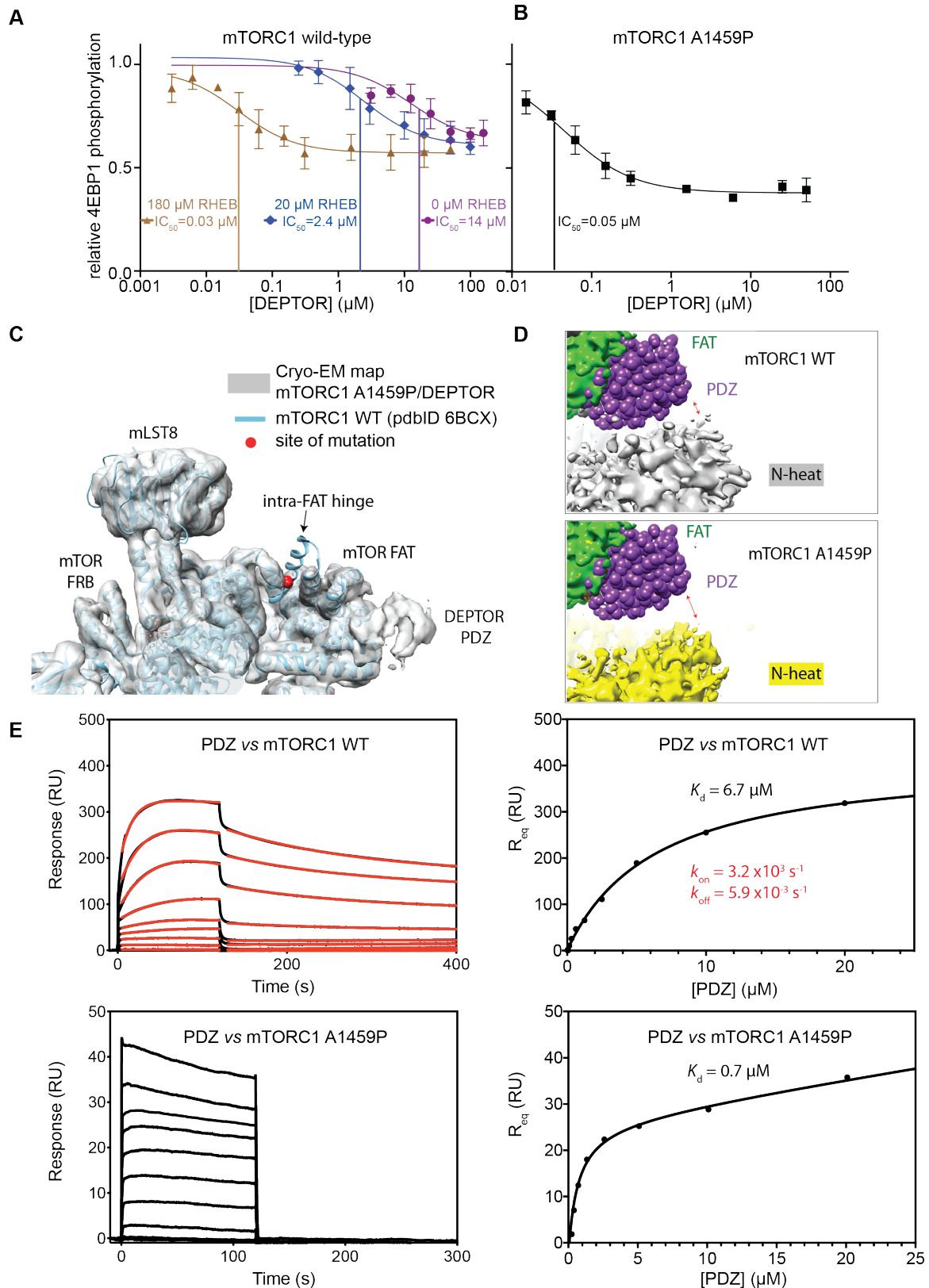
1 (residues 228-409) vs. free FRB in a ^1H - ^{15}N BEST-TROSY experiment. The CSPs are
2 displayed on the ribbon model of mTORC1 that is shown together with the EM density
3 (semitransparent gray) for the FRB region of the mTORC1/DEPTOR complex. The EM
4 density corresponding to DEPTOR is colored purple. See Figure 5-figure supplement 1A-C for
5 NMR spectra and analysis.

6 **(B)** Mapping the DEPTOR linker regions that interact with the FRB domain by the chemical
7 shift perturbation of the FRB-bound linker vs. free linker in a ^1H - ^{15}N BEST-TROSY
8 experiment. See Figure 5-figure supplement 1D for NMR spectra.

9 **(C)** Mapping the DEPTOR regions interacting with the mTOR FRB investigated by HDX-MS.
10 Reduction in HDX in full-length DEPTOR in the presence of the FRB suggests that regions of
11 the linker interact with the FRB. Four different coloured lines represent 4 time points (3 s =
12 orange, 30 s = red, 300 s = blue, 3000 s = black), the grey coloured area represents the standard
13 error. See also Supplementary File 1.

14 **(D)** Interaction of the PDZ domain with a portion of a DEPTOR linker revealed from a
15 comparison of DEPTOR PDZ (residues 324-409) and linker-PDZ (residues 228-409) ^1H - ^{15}N
16 BEST-TROSY spectra. The weighted chemical shift perturbation is calculated between the
17 nearest linker-PDZ peak to the assigned PDZ peak in the overlaid spectra (see Figure 5-figure
18 supplement 2A). The minimal map coloured from green to red shows the interaction of the
19 linker with the surface of the PDZ.

20 **(E)** Stability of long-linker-PDZ (residues 228-409) and short-linker-PDZ (305-409) is greatly
21 improved over the PDZ domain alone (residues 324-409), as shown by ~ 10 °C increase in the
22 protein melting temperature measured by differential scanning fluorimetry.



1

2 **Figure 6. DEPTOR inhibits activated mTORC1 more strongly than basal mTORC1, but**
 3 **phosphorylated DEPTOR does not inhibit mTORC1.**

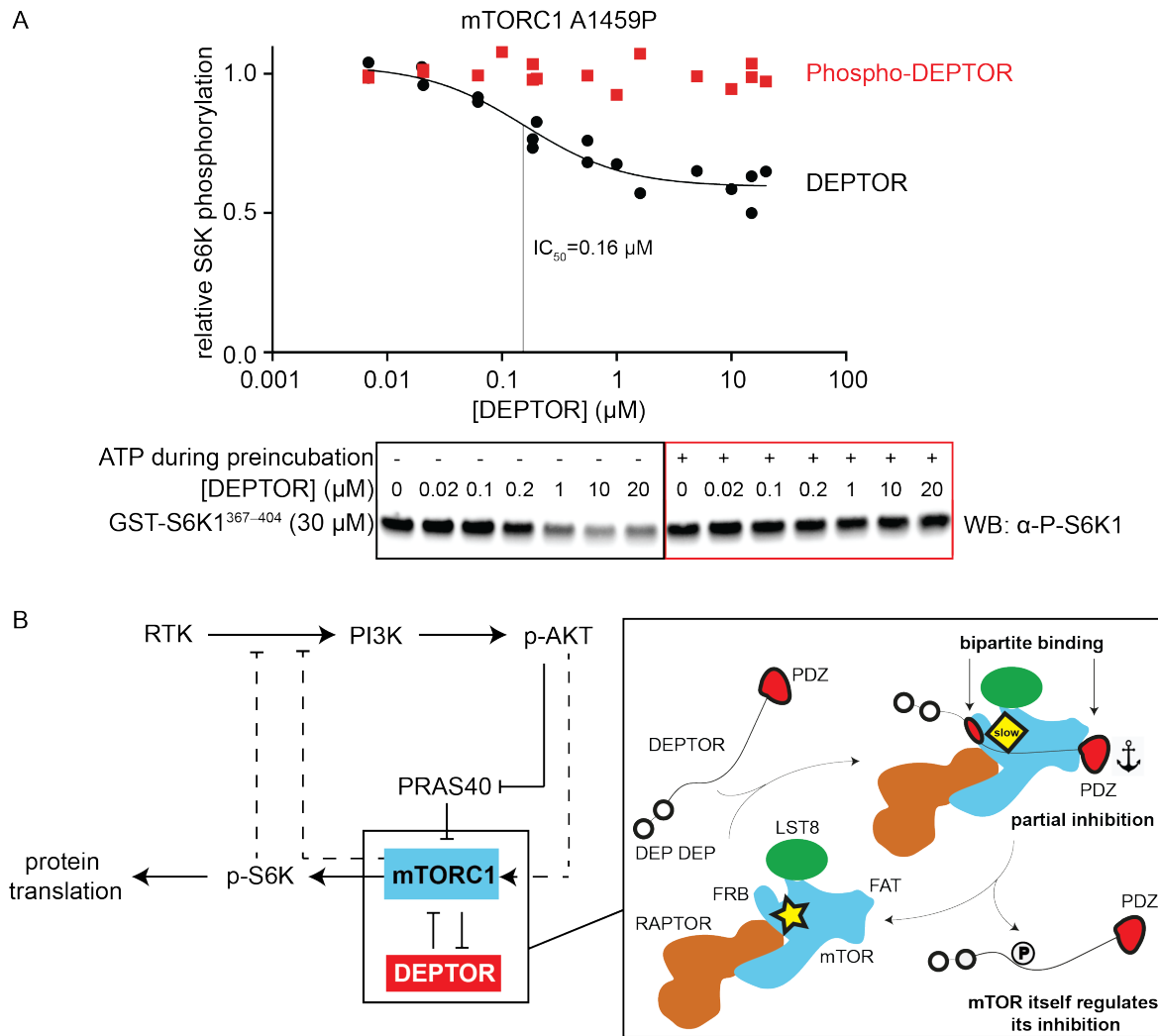
1 (A) Increasing concentrations of RHEB-GTP lead to activated mTORC1 and at the same time
2 result in a decreased DEPTOR IC₅₀ (substrate was 4EBP1). Band intensities reflecting P-
3 4EBP1 were normalized to the control (0 μM DEPTOR) and the data (mean ± SD, n ≥ 3) was
4 fit by a nonlinear regression to determine IC₅₀.

5 (B) Cancer-associated, hyperactive mutant mTORC1-A1459P also shows a decreased
6 DEPTOR IC₅₀. Band intensities reflecting P-4EBP1 were normalized to the control (0 μM
7 DEPTOR) and the data (mean ± SD, n ≥ 3) was fit by a nonlinear regression to determine IC₅₀.

8 (C) Cryo-EM reconstruction of A1459P mTORC1 mutant and DEPTOR reveals the loss of an
9 mTOR helix at the mutation site. The site of mutation lies within the hinges of mTORC1 which
10 are involved in introducing major conformational changes in mTOR upon RHEB-induced
11 activation.

12 (D) Alignment of WT and mutant A1459 mTORC1 on the PDZ-binding site in the FAT domain
13 (shown with green density bound to the PDZ domain illustrated with magenta spheres) reveals
14 a shift of mTOR N-heat domain for mTORC1 A1459P/DEPTOR (yellow density) with respect
15 to mTORC1 WT/DEPTOR (grey density). This shift increases the crevice between the FAT
16 and the N-heat domains in mTORC1 A1459P and creates an easier access for DEPTOR PDZ
17 to its binding site.

18 (E) Comparison of PDZ binding to wild-type and mutant mTORC1 analysed by SPR. PDZ
19 (construct 305-409) binds to wild-type mTORC1 with slow on/off kinetics and lower affinity,
20 whereas it binds the mutant mTORC1, with fast on/off kinetics and 10-fold greater affinity.
21 The total binding data were fit to a model with one-site specific binding combined with a
22 linear non-specific component.



1

2 **Figure 7. mTORC1 directly controls the function of its negative regulators.**

3 (A) DEPTOR pre-phosphorylated by activated mTORC1 (phospho-DEPTOR) shows no
 4 inhibition of mTORC1 activity. Band intensities reflecting P-S6K³⁶⁷⁻⁴⁰⁴ peptide were
 5 normalized to the control (0 μM DEPTOR). Each data point from three independent
 6 experiments is shown with varied [DEPTOR] in each experiment and results were fit by a
 7 nonlinear regression to determine IC_{50} .

8 (B) Negative feedback to PI3K (dashed lines) controls the activity of the two mTORC1
 9 inhibitors PRAS40 and DEPTOR. If mTORC1 inhibition is high, PI3K is activated due to a
 10 loss of the negative feedback. Increased p-AKT results in decreased PRAS40 inhibition, with
 11 increased mTORC1 activity, which in turn reduces DEPTOR inhibition. mTORC1 activity

1 alone is sufficient to reduce DEPTOR inhibition, without the necessity of DEPTOR's
2 ubiquitination and subsequent degradation. This increased mTORC1 activity in turn induces
3 negative feedback to PI3K and prevents an overactivation of this pathway, keeping activities
4 balanced.
5

1 **Materials and Methods**

2 **Resource Availability**

3 Further information and requests for resources and reagents should be directed to and will be
4 fulfilled by the Lead Contact, Roger L. Williams (rlw@mrc-lmb.cam.ac.uk).

5 The cryo-EM map and the model are deposited with the EMDB and PDB, respectively.
6 Backbone assignments of DEPTOR PDZ, mTOR-FRB and DEPTOR linker have been
7 submitted to the BMRB, Biological Magnetic Resonance Bank with the accession numbers
8 50324, 50325 and 50326, respectively.

9

10 **Experimental Model and Subject Details**

11 *E. coli* C41(DE3)-RIPL cells were used for expression of all DEPTOR constructs as well as
12 for 4EBP1, S6K1, RHEB and the isolated mTOR FRB domain. *E. coli* LOBSTR cells
13 (EC1001, KeraFast) were used for the expression of PRAS40. The cells were grown in 2xTY
14 media at 37 °C, induced at the OD₆₀₀ = 0.7 with 0.3 mM isopropyl-d-1-thiogalactopyranoside,
15 followed by 16 h of growth at 18 °C before harvest. Expi293F cells (Thermo Fisher A14527,
16 RRID:CVCL_D615) were used for the production of mTORC1 and its mutants. Cells were
17 grown in a Multitron Pro shaker set at 37 °C, 8% CO₂ and 125 rpm. Cells were transfected at
18 a cell density of 2.5x10⁶ cells/mL by co-transfecting plasmids (1.1 mg total DNA/L cells) using
19 PEI (Polyethyleneimine "MAX", MW 40,000, Polysciences, 24765, total 3 mg PEI/L cells).

20

21 **Method Detail**

22 *Recombinant protein expression and purification*

23 For DEPTOR (WT), DEPTOR 13S/T-A, DEPDEP, DEPDEP linker and linker-PDZ, a cell
24 pellet of a 12 L *E. coli* culture was resuspended in 75 mL of lysis buffer (50 mM Tris pH 8,
25 100 mM NaCl, 1 mM TCEP). Two Complete EDTA-free inhibitor tablets (Roche), 500 µL of

1 a 100 mM PEFA solution and 40 mg of lysozyme were added to the cell suspension, which
2 was subsequently sonicated and centrifuged at 35000 rpm for 35 min. The resulting supernatant
3 was incubated with 3 mL equilibrated Glutathione-Sepharose 4B beads (GE Healthcare) for 45
4 min at 4°C, while rolling at 18 rpm. Sedimentation of the beads for 2 min at 600 g and the
5 careful removal of the unbound fraction was followed by extensive washing with lysis buffer
6 under gravity flow conditions. Bound DEPTOR was incubated with TEV protease overnight
7 at 4°C. Cleaved DEPTOR protein was then collected and applied to a 5 mL HiTrap Heparin
8 HP column (GE Healthcare) equilibrated with HEP-A buffer (50 mM HEPES pH 8, 100 mM
9 NaCl, 1 mM TCEP). After washing the column with 80 mL of HEP-A buffer, DEPTOR was
10 eluted with a gradient using HEP-B buffer (50 mM HEPES pH 8, 450 mM NaCl, 1 mM TCEP).
11 DEPDEP was purified via a 5 mL HiTrap Q column (GE Healthcare) in place of the Heparin
12 column using identical buffer composition. A final size-exclusion chromatography step on a
13 S75 16/60 column was performed (50 mM HEPES pH 8, 100 mM NaCl, 1 mM TCEP) and
14 fractions were analysed by SDS-PAGE. DEPTOR-containing fractions were combined and
15 concentrated to 23 mg/mL. The purified DEPTOR protein was flash frozen in liquid nitrogen
16 and stored at -80°C.

17 After cell lysis as described for DEPTOR (WT), the His-lipoyl-tagged DEPTOR PDZ
18 was loaded onto a 5 mL NiNTA column and washed with 75 mL lysis buffer containing 10
19 mM imidazole prior to eluting the protein with 25 mL lysis buffer spiked with 300 mM
20 imidazole. TEV cleavage was performed overnight as described above. The buffer salt
21 concentration was diluted to 30 mM NaCl and the solution was flown through a 5 mL HiTrap
22 Q HP (GE Healthcare) and a NiNTA column. The flowthrough was collected and concentrated
23 before gel filtration as described for DEPTOR (WT).

24 mTORC1 complexes were expressed by transient transfection of Expi293F cells grown
25 in Expi293 media. A total of 1.1 mg DNA/L cells was co-transfected into cells at a density of

1 2.5 x10⁶ cells/mL using PEI (Polyethyleneimine "MAX", MW 40,000, Polysciences, 24765,
2 total 3 mg PEI/L cells). After 48 h, cells were harvested by centrifugation and cell pellets were
3 frozen in liquid N₂. Cell pellet from 2 L culture was resuspended in 200 mL of the lysis buffer
4 (50 mM Tris-HCl, pH 8, 500 mM NaCl, 10% glycerol, 1 mM TCEP, 1 mM EDTA, 1 mM
5 EGTA), supplemented with six Complete EDTA-free) protease-inhibitor tablets (Roche), 5 µL
6 Universal nuclease (Pierce, 250U/uL), and 400 µL of a 100 mM PEFA solution, using a
7 Dounce homogenizer (Kontes, 100 mL, Pestle B, small clearance) on ice and sonication (2x 15
8 s ON at 40% amplitude). The cell lysate was spun at 15000 rpm for 35 min in a Ti45 rotor,
9 then filtered through Minisart 5 µm filter. Two tandem Strep-Trap HP columns (GE Healthcare
10 28-9075-48) were equilibrated with lysis buffer, then the filtered lysate was loaded onto the
11 column at a flow of 2.5 mL/min. Extensive washes with lysis buffer (>200 mL), with 50 mL
12 lysis buffer supplemented with 200 mM Li₂SO₄, and 50 mL of the TEV cleavage buffer (50
13 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM TCEP) were performed prior to
14 loading 0.1 mg/mL TEV protease onto the column. The cleavage reaction was performed on
15 the column overnight. The protein was then eluted and the salt concentration was adjusted to
16 50 mM NaCl. The protein was then loaded onto a 5 mL HiTrap Q column (GE Healthcare)
17 equilibrated with 50 mM HEPES, pH 7.5, 50 mM NaCl, and was eluted via a salt gradient.
18 mTORC1-containing fractions were concentrated using an Amicon Ultra-4 100 kDa
19 concentrator, spinning in 3 min intervals at 1,000 rcf. Gel filtration was performed using a
20 Superose 6 Increase (10/300) column in gel filtration buffer (50 mM HEPES, pH 7.5, 200 mM
21 NaCl, 1 mM TCEP). mTOR complex fractions were pooled and again concentrated using an
22 Amicon Ultra-4 100kDa concentrator before freezing the protein.

23 Human RHEB was cloned into a pOPTG vector encoding an N-terminal GST-tag
24 followed by a TEV cleavage site for proteolytic removal. The fusion protein was overexpressed
25 in *E. coli* C41(DE3)RIPL cells as described for DEPTOR. Cells were lysed in 30 mM Tris pH

1 8.0, 200 mM NaCl, 0.3 mM TCEP, 1.5 mM MgCl₂ and 5% glycerol and GST-fused RHEB
2 was purified by an affinity chromatography step on Glutathione-Sepharose 4B beads. The
3 GST-tag was removed by incubating with TEV protease overnight. To separate the HIS6-TEV
4 protease from the RHEB protein, a nickel affinity chromatography step was performed before
5 a final gel filtration step on a Superdex 75 16/60 column with 30 mM Tris pH 8.0 (4 °C), 100
6 mM NaCl, 5 mM MgCl₂ and 1 mM TCEP, which yielded purified RHEB. Removal of bound
7 GDP was achieved by incubating the protein for 1 h on ice with EDTA buffer containing 20
8 mM HEPES pH 7, 100 mM NaCl, 20 mM EDTA and 1 mM TCEP. Next, the buffer was
9 exchanged to 50 mM HEPES pH 7, 100 mM NaCl, 5 mM MgCl₂, 1 mM TCEP using an
10 Amicon Ultra-4 4 kDa concentrator before concentrating the protein to 15.5 mg/ml. Purified
11 RHEB was flash frozen in liquid nitrogen and stored at -80 °C.

12 Human PRAS40 was purified as described previously (Anandapadamanaban et al.,
13 2019).

14

15 *mTORC1 activity assays*

16 All reactions were performed in kinase buffer (KB) consisting of 25 mM HEPES, pH 7.4, 75
17 mM NaCl, 0.9 mM TCEP, 5% glycerol, at 30 °C for a duration of 45 min for non-activated
18 mTORC1, 2 to 4 min for activated mTORC1, and at 20 °C for 20 min for the unstable DEPTOR
19 PDZ construct using non-activated mTORC1. Reactions were set up by preincubating 100 nM
20 non-activated mTORC1 with either 5 μM 4EBP1, 10 μM TOS-less 4EBP1 or 30 μM GST-
21 S6K1³⁶⁷⁻⁴⁰⁴ peptide as substrates and various concentrations of the inhibitors for 10 min on ice.
22 After 30 s temperature equilibration at 30 °C, reactions were started by the addition of 75 μM
23 ATP and 10 mM MgCl₂. For the activated mTORC1 complexes (mTOR-A1459P mutant or in
24 the presence of RHEB-GTP), 30 μM 4EBP1 or GST-S6K1³⁶⁷⁻⁴⁰⁴ peptide and 20 nM mTORC1
25 were used and the reaction was started with 500 μM ATP and 10 mM MgCl₂. For RHEB-

1 activated mTORC1, RHEB was preincubated for 1 h with a 30-fold molar excess of GTP γ S
2 (Jena Bioscience NU-412-20, lot IT008-18). Next, mTORC1, DEPTOR, 4EBP1 and RHEB-
3 GTP γ S were mixed in the order of mentioning and preincubated for 10 min on ice. After 30 s
4 temperature equilibration at 30 °C, reactions were started by the addition of 500 μ M ATP and
5 10 mM MgCl₂. All reactions were quenched with 2x SDS sample buffer and resolved on a 4-
6 12% NuPage Bis-Tris gel. Western blots were performed using a 0.2 μ M pore size
7 nitrocellulose membrane (Invitrogen IB301002) and the iBlot dry blotting transfer system
8 (Invitrogen). Blocking was performed using 5% Marvel in TBST buffer (100 mM Tris-HCl,
9 150 mM NaCl, 0.1 % Tween 20). Antibodies were obtained from Cell Signalling (P-4EBP1
10 (T37/46) Rabbit AB, 9459L, RRID:AB_330985; 4EBP1 Rabbit AB, 9452S,
11 RRID:AB_331692; P-p70 S6 Kinase (T389) Rabbit, 9205L, RRID:AB_330944; Anti-Rabbit
12 IgG, HRP-linked Antibody, 7074, RRID:AB_2099233). Antibody solutions contained 5%
13 BSA in TBST, using 1:1000 dilution for primary antibodies and 1:5000 for the secondary
14 antibody. Detection was performed using the Bio-Rad ChemiDoc-Touch Imaging System.

15 For the inhibition of mTORC1 A1459P mutant by DEPTOR measured by Phos-tag
16 SDS PAGE, the reactions containing 30 nM mTORC1, 15 μ M 4EBP1, 250 mM ATP, 10 mM
17 MgCl₂ and varied concentration of DEPTOR were incubated for 4 min at 30 °C and then
18 quenched by the addition of 2X SDS sample buffer. The samples were analysed by SuperSep
19 Phos-tag (50 μ M), 7.5% precast gels (192-17381), with MOPS –EDTA running buffer
20 supplemented with 5 mM sodium bisulphate. Staining was done using InstantBlue and
21 quantification followed using the Bio-Rad ChemiDoc-Touch Imaging System.

22 To analyse phosphorylation of DEPTOR by mTORC1, assays contained 30 nM
23 mTORC1-A1459P mutant, 250 μ M ATP, 10 mM MgCl₂ and 20 μ M of each DEPTOR variant.
24 Phosphorylation was detected by SuperSep Phos-tag precast gels using InstantBlue stain and
25 Bio-Rad ChemiDoc-Touch Imaging System.

1 To compare the inhibition by DEPTOR vs. phospho-DEPTOR, reactions were set up
2 using a dilution series of DEPTOR (30 - 0 μ M) and 60 nM mTORC1-A1459P mutant. Reaction
3 mix was split in two, one reaction was started by adding 20 mM MgCl₂ and 500 μ M ATP (to
4 produce phospho-DEPTOR), the second mix was spiked with an equal volume of KB
5 (DEPTOR as a control). After 2 h, an aliquot of each reaction was quenched in SDS buffer and
6 analysed by Phos-tag gel for the completeness of mTORC1 dependent DEPTOR
7 phosphorylation (\geq 80 %). At the same time, a second aliquot of each reaction was added into
8 substrate mix resulting in a final concentration of 30 μ M GST-S6K, 500 μ M ATP, and 20 mM
9 MgCl₂ in KB to test for inhibition of phospho-DEPTOR vs. DEPTOR. The reaction was
10 quenched after 2 min, and the results were analysed as described above by western blots.

11 All IC₅₀ were determined by the non-linear regression $y=y_{\min} + (y_{\max}-$
12 $y_{\min})/(1+([DEPTOR]/IC_{50}))$. $K_{M,4EBP1}$ in the presence of DEPTOR was fit according to the
13 Michaelis Menten equation $y = v_{\max}*[4EBP1]/(K_M + [4EBP1])$. Statistical analysis and curve
14 fitting for IC₅₀ experiments was performed in GraphPad Prism (RRID:SCR_002798). Each
15 individual run was normalized to [inhibitor] = 0 μ M. Standard deviation of at least three
16 independent replicates is shown for each data point in the graph. Standard deviation for the fit
17 IC₅₀, for the residual activity (y_{\min}) and for $K_{M,4EBP1}$ is reported in the figures.

18

19 *Cryo-EM sample preparation*

20 Purified wild-type mTORC1 (0.7 μ M) and DEPTOR (1.4 μ M) were mixed in \sim 300 μ L and
21 incubated for 30 min on ice. Following the addition of 0.02% glutaraldehyde (Sigma G5882)
22 from a 1% glutaraldehyde stock in GraFix buffer A (50 mM HEPES pH 7.5, 0.1 M NaCl, 1
23 mM TCEP, 10% glycerol), the sample was immediately subjected to a gradient fixation
24 (GraFix) (Kastner et al., 2008). For that, the sample was loaded on a 12 mL gradient of 10-
25 30% glycerol and 0-0.2% glutaraldehyde in 50 mM HEPES pH 7.5, 0.1 M NaCl, 1 mM TCEP)

1 performed in a SW40 rotor tube (Ultra-Clear, Beckman 344060) using a gradient maker
2 (Biocomp Instruments). The sample was centrifuged in a SW40 rotor (Beckman) at 33,000
3 rpm for 14 h. After centrifugation, 0.45 mL fractions were collected manually from top of the
4 tube, analysed by SDS-PAGE, and the fractions containing crosslinked material were pooled,
5 quenched by the addition of final 50 mM Tris pH 7.5 and concentrated to 250 μ L using an
6 Amicon Ultra-15 100 kDa concentrator. Cross-linked mTORC1/DEPTOR was further run on
7 a Superose 6i 10/300 column equilibrated in 50 mM HEPES pH7.5, 200 mM NaCl, 0.3%
8 CHAPS and 1 mM TCEP and the peak fractions were concentrated to 0.11 OD₂₈₀ used
9 immediately for cryo-EM grid preparation.

10 Purified mutant mTORC1 A1459P (1.2 μ M) and DEPTOR (6.8 μ M) were preincubated in the
11 presence of 1 mM MgCl₂ and 500 μ M AMP-PNP (Jena Biosciences) for 20 min and used for
12 cryo-EM grid preparation.

13

14 *Cryo-EM data collection and processing*

15 Holey carbon Quantifoil Au R 1.2/1.3 (300 mesh) grids were glow-discharged using an
16 Edwards Sputter Coater S150B for 60 s at 40 mA. The grids were covered with graphene oxide
17 as previously described (Boland et al., 2017). In short, 3 μ L of a 0.2 mg/mL graphene oxide
18 dispersion (Sigma, cat number 777676) was added to the carbon side of the grids and incubated
19 for 1 min. Excess of graphene oxide solution was removed by blotting with Whatman No.1
20 filter paper and washed three times with Milli-Q H₂O before air-drying for 5 min at room
21 temperature. Graphene oxide covered grids were stored in a grid box overnight.

22 A 3 μ L aliquot of freshly-prepared crosslinked mTORC1/DEPTOR complex at OD₂₈₀
23 = 0.11 was added to graphene oxide-covered grids and blotted for 11-13 s at 4 °C and then
24 plunge-frozen in liquid ethane using a custom-fabricated manual plunger (MRC Laboratory of
25 Molecular Biology). A total of 2370 micrographs of the human mTORC1/DEPTOR complex

1 were acquired on a FEI Titan Krios electron microscope operated at 300 keV. Zero-energy loss
2 images were recorded on a Gatan K2 Summit direct electron detector operated in super-
3 resolution mode with a Gatan GIF Quantum energy filter (20 eV slit width) using SerialEM
4 (Mastrorade, 2005) for automated collection. Images were recorded at a calibrated
5 magnification of 34,965 (pixel size of ~ 1.43 Å) with a dose rate of ~ 2.5 electrons/Å²/s. An
6 exposure time of 16 s was fractionated into 20 movie frames adding to a total dose of 40
7 electrons/Å². For data collection, a defocus-range was set to -1.6 to -3.2 μm (Table 1).

8 A 3 μL aliquot of mutant mTORC1 A1459P/DEPTOR complex was added onto
9 UltrAuFoil R 1.2/1.3 Au 300 mesh (Quantifoil Micro Tools GmbH) after the grid was glow-
10 discharged using an Edwards Sputter Coater S150B for 60 s at 40 mA. Plunge freezing was
11 performed using Vitrobot (Thermo Fisher Scientific) with a blotting time of 2 s at 14 °C and
12 95% humidity and a force of -15. A total of 4759 micrographs of the mutant mTORC1 A1459P
13 /DEPTOR complex were acquired on a FEI Titan Krios electron microscope operated at 300
14 keV. Zero-energy loss images were recorded on a Gatan K3 Summit direct electron detector
15 operated in super-resolution mode with a Gatan GIF Quantum energy filter (20 eV slit width)
16 using EPU (Thermo Fisher Scientific) for automated collection. Images were recorded at a
17 calibrated magnification of 81,000 (pixel size of ~ 1.1 Å) with a dose rate of ~ 1.12
18 electrons/Å²/s. An exposure time of 3.25 s was fractionated into 50 movie frames adding to a
19 total dose of 56 electrons/Å². For data collection, a defocus-range was set to -1.4 to -3.0 μm.

20

21 *Image processing*

22 All image-processing steps were done using the RELION 3 software package ((Scheres,
23 2012), RRID:SCR_016274), which includes Getf (Zhang, 2016), MotionCor2 (Zheng et al.,
24 2017) and ResMap (Kucukelbir et al., 2014). Micrographs were processed using GPU-
25 accelerated MotionCor2 to correct for electron beam-induced sample motion, while contrast

1 transfer function (CTF) parameters were determined using Gctf. Reference-based autopicking
2 was performed on the full dataset using Relion3 with initial templates obtained from a previous
3 mTORC1/DEPTOR dataset.

4 For the wild-type mTORC1/ DEPTOR data set, 491,404 particles were extracted with
5 a particle box size of 400 by 400 pixels. Two rounds of reference-free 2D classification (using
6 a mask with a diameter of 350 Å) resulted in a selection of 390,636 particles. This set of
7 particles was subjected to a 3D classification over 30 iterations in point group C_1 using a low-
8 pass filtered (50 Å) ab-initio reference which was created using the SGD algorithm for de-novo
9 3D model generation introduced in Relion3. Selection of reasonably looking classes by
10 visualisation in Chimera (RRID:SCR_004097) and by paying attention to the rotational and
11 translational accuracies for six classes reduced the number of particles to 333,462 sorted into
12 five 3D classes. Without providing a mask around the mTORC1/DEPTOR complex, the
13 subsequent 3D auto-refinement of these particles applying C_2 symmetry led to a reconstruction
14 of 6.7 Å resolution, based on the gold-standard FSC = 0.143 criterion (Rosenthal and
15 Henderson, 2003; Scheres, 2012).

16 To correct for beam-induced particle movements, increase the signal-to-noise ratio for
17 all particles, and to apply radiation-damage weighting, the refined particles were further
18 'polished' using the Bayesian approach implemented in Relion3. Following this step, another
19 3D autorefinement in C_2 using a mask around the mTORC1/DEPTOR complex as well as
20 applying solvent-flattened FSCs yielded a reconstruction of 4.3 Å resolution (FSC = 0.143
21 criterion). After a CTF- and beamtilt-refinement for the estimation of per-particle defoci and
22 beamtilt values for the complete set of selected particles, a subsequently performed 3D
23 autorefinement resulted in a mTORC1/DEPTOR reconstruction of 4.2 Å resolution (FSC =
24 0.143 criterion). A similar processing strategy was performed for the mutant mTORC1

1 A1459P/DEPTOR complex data set, comprising a total of 97,314 particles and resulting in a
2 reconstruction of 4.7 Å resolution.

3 To improve resolution (especially in the DEPTOR region), we expanded the wild-type
4 mTORC1/DEPTOR dataset using the `reliion_particle_symmetry expand` command while
5 applying C2 symmetry and performed focussed classification with signal subtraction (Bai et al.,
6 2015). This strategy yielded higher resolution in previous mTORC1 structures
7 (Anandapadamanaban et al., 2019; Yang et al., 2017). Using focussed 3D-classification with
8 signal subtraction on the expanded 666,924 monomer particles without image alignment
9 yielded two reasonable classes with a total of 464,013 monomers. A 3D refinement of this set
10 of particles corresponding to monomer density led to a reconstruction of 4.4 Å, based on the
11 gold-standard FSC = 0.143 criterion.

12 To improve the density map of DEPTOR PDZ region, we performed another focused
13 classification with signal subtraction on the monomer particles. A mask was applied to the
14 region of interest on the PDZ (DEPTOR residues 324-409) and surrounding mTOR domains
15 (N-heat and FAT domain, 61-903 and 1474-1644, respectively), particles were 3D classified
16 without image alignment, and the best class was selected for further refinement of the original
17 (unmasked) particles. This resulted in smaller subsets of the original 223,576 particles; in
18 which the PDZ density was better defined. A 3D refinement of the above selected particles
19 resulted in a map at an overall 4.3 Å resolution, based on the gold-standard FSC = 0.143
20 criterion (Figure 3-figure supplement 2H).

21

22 *Cryo-EM model building and refinement*

23 After correction for the detector modulation transfer function (MTF) and B-factor
24 sharpening, the post-processed map was used for inspection in Chimera (Pettersen et al., 2004)
25 and model building in Coot (Casanal et al., 2020; Emsley and Cowtan, 2004). Superimposing

1 basal-state monomer and RHEB-activated monomer models of mTORC1 taken from
2 previously reported structures (PDB 6BCX and 6BCU, respectively), it was apparent that the
3 6BCX agreed better with the mTORC1/DEPTOR density. The 6BCX model was broken into
4 domains that were rigidly fit with COOT to the observed density. The model was manually fit
5 to the density using ISOLDE (Croll, 2018) in ChimeraX ((Pettersen et al., 2020),
6 RRID:SCR_015872). The CS-Rosetta model for the DEPTOR PDZ domain was manually fit
7 to the density. Backbone dihedral angles were predicted for each residue using Talos-N (Shen
8 and Bax, 2013). These were used as external torsion angle restraints for real-space refinement
9 in COOT (RRID:SCR_014222) and for REFMAC5 ((Brown et al., 2015; Murshudov et al.,
10 1997), RRID:SCR_007255). The model was refined in REFMAC with external (PDB 6BCX)
11 restraints for mTORC1 from PROSMART (Kovalevskiy et al., 2016; Nicholls et al., 2017) and
12 self, H-bond and Talos-N restraints for the PDZ domain. Manual building and refinement were
13 iterated. Local resolutions were estimated using ResMap (Figure 3-figure supplement 2E, G).

14 For the A1459P mutant mTORC1 in a complex with DEPTOR, the wild-type monomer
15 mTORC1 was used as an initial model and placed into a monomer density in the A1459P
16 mTORC1 cryo-EM map with C2 symmetry. The model was adjusted manually and refined
17 using ISOLDE. The ISOLDE-refined monomer was further refined in REFMAC5 using the
18 C2 dimer density, with strict C2 symmetry constraints and with the wild-type monomer for
19 external restraints.

20

21 *Multibody refinement analysis of wild-type mTORC1/DEPTOR particles*

22 Multibody refinement analysis was performed following the protocol described
23 previously (Nakane et al., 2018). The wild-type mTORC1/ DEPTOR complex was split into
24 four bodies of >100 kDa each via the Segger tool in Chimera (Figure 6-figure supplement 1B)
25 (Pintilie et al., 2010). They were arbitrarily named ‘mTOR’, ‘RAPTOR’, ‘M-heat’, and ‘N-

1 heat', though they do not correspond to these exact domain boundaries. Corresponding masks
2 were created using 15 Å lowpass filtered maps and soft-edges with a width of 20 Å. All bodies
3 were set to rotate relative to the 'mTOR' body by 10 or 15 degree, 'mTOR' was set to rotate
4 relative to the 'M-heat', and the standard deviations on the body translations were all set to 2
5 to 3 pixels (2.8 – 3.2 Å). With the relion_flex_analyse program two separate STAR files with
6 10,558 particles and 13,975 particles were created, for which the amplitude along the third
7 eigenvector is less than -7, or greater than 7, respectively (Figure 6-figure supplement 1B).
8 Separate refinements of these subsets yielded maps with overall resolution estimates of 8.3 and
9 7.6 Å, respectively.

10

11 *NMR sample preparation*

12 After transformation and overnight growth in 50 mL 2xTY media, 20 mL of the starter
13 culture were spun down at 3000 g and washed with water. The cell pellet was resuspended in
14 1 L M9 minimal media containing 1 g/L ¹⁵NH₄Cl, 4 g/L ¹³C-glucose (for double-labelled
15 samples) or ¹²C-glucose (for single-labelled samples), 1.7 g/L Yeast Nitrogen Base Without
16 Amino Acids and Ammonium Sulfate (Y1251 Sigma: 2 µg/L Biotin, 400 µg/L Calcium
17 pantothenate, 2 µg/L Folic acid, 400 µg/L Niacin, 200 µg/L p-Aminobenzoic acid, 400 µg/L
18 Pyridoxine HCl, 200 µg/L Riboflavin, 400 µg/L Thiamine HCl, 2 mg/L Inositol, 500 µg/L
19 Boric acid, 40 µg/L Copper sulfate, 100 µg/L Potassium iodide, 200 µg/L Ferric chloride, 400
20 µg/L Manganese sulfate, 200 µg/L Sodium molybdate, 400 µg/L Zinc sulfate, 1 g/L Potassium
21 phosphate monobasic, 0.5 g/L Magnesium sulfate, 0.1 g/L Sodium chloride, 0.1 g/L Calcium
22 chloride, and 100 µg/mL Ampicillin. Cells were induced by the addition of 1 mM IPTG once
23 OD₆₀₀ = 0.8 was reached and grown with shaking at 220 rpm at 23 °C for 16 h, harvested and
24 stored at -80 °C.

25 DEPTOR PDZ and linker-PDZ for the NMR experiments were purified as described above.

1 The isolated mTOR FRB domain (residues 2015-2114) was purified from a 6 L culture
2 of C41(DE3)RIPL cells transformed with plasmid pOPL107, grown to OD₆₀₀ = 0.8 and
3 induced with 0.3 mM IPTG at 16 °C for 18 h. Cells were lysed by sonication in a GST-A buffer
4 (50 mM Tris pH 8, 100 mM NaCl, 1 mM TCEP) supplemented with 0.25 mg/ml lysozyme and
5 2 μL/100 ml of Universal nuclease. Following ultracentrifugation at 35 k rpm in Ti45 rotor for
6 35 min, the supernatant was purified by affinity chromatography on the Glutathione-Sepharose
7 4B beads (GE Healthcare, GE17-0756-05) equilibrated in GST-A buffer. The GST-FRB fusion
8 was eluted with 20 mM glutathione in the same buffer. The eluate was diluted with 2 volumes
9 of dilution buffer (50 mM Tris pH 8, 1 mM TCEP), loaded on a 5 ml HiTrapQ column and
10 eluted with a 0-1M NaCl gradient. The fractions containing GST-FRB were concentrated in a
11 30K Ultra-15 concentrator and further purified by gel filtration on a Superdex 75 16/60 column
12 equilibrated in 50 mM HEPES pH 8, 100 mM NaCl, 1 mM TCEP.

13 The DEPTOR linker (residues 228-323) was purified from a 6 L culture of
14 C41(DE3)RIPL cells transformed with plasmid pOPL159, grown to OD₆₀₀ = 0.8 and induced
15 with 0.3 mM IPTG at 16 °C for 18 h. The His₆-tagged protein was purified by affinity
16 chromatography on Ni-NTA agarose beads (Qiagen 30230) and cleaved on beads with TEV
17 protease o/n at 4 °C. The cleaved protein was diluted with 1 volume of 50 mM Tris pH8, 1 mM
18 TCEP (to achieve ~ 50 mM NaCl) and loaded on a 5 mL HiTrapQ column equilibrated in 50
19 mM HEPES pH8, 25 mM NaCl, 1 mM TCEP. The flow-through fraction containing DEPTOR
20 linker was concentrated in a 3K Amicon Ultra-15 concentrator and further purified by gel
21 filtration on Superdex 75 16/60 column equilibrated in 50 mM HEPES pH 8, 100 mM NaCl, 1
22 mM TCEP.

23

24 *DEPTOR PDZ homology model generation by NMR*

1 All NMR data sets were collected at 278K using Bruker Avance II+ 700 MHz or
2 600MHz Avance III spectrometers with TCI triple resonance cryoprobes unless otherwise
3 stated. All samples were prepared with 5% D₂O as a lock solvent, at pH 8 with 50 mM HEPES
4 and 200 mM NaCl.

5 ¹H-¹⁵N BEST-TROSY (band selective excitation short transients-transverse relaxation
6 optimized spectroscopy) were collected for all samples using an optimised pulse sequence
7 (Favier and Brutscher, 2011). The assignment of backbone H_N, N and C_α,C_β resonances of the
8 190 μM ¹³C/¹⁵N DEPTOR PDZ (residues 364-409) sample was completed using the following
9 3D datasets acquired as pairs to provide own and preceding carbon connectivities. In most
10 cases the amide proton and nitrogen dimension were taken from the ¹H-¹⁵N BEST-TROSY
11 experiment: HNCO, HN(CA)CO, CBCA(CO)NH and HNCACB which were used as
12 experimental pairs with 1024 complex points in the proton, and 64, 110 complex points in the
13 nitrogen and carbon dimensions, respectively. Partial H_α, H_β side-chain chemical shift
14 assignments were obtained from an HBHA(CO)NH spectrum collected with 1024, 64 and 110
15 complex points in the proton, nitrogen and the second proton dimensions, respectively.
16 Assignment of the carbon side-chain resonances was completed with HC(C)H- and (H)CCH-
17 TOCSY experiments (also collected with 1024, 64 and 110 points). These assignments enabled
18 the analysis of a limited set of through space connectivities from ¹⁵N and ¹³C edited NOESY
19 experiments both acquired with a mixing time of 120 ms and collected with 2048, 60/80, and
20 160 in the proton, nitrogen/carbon, and second proton dimensions, respectively. All data was
21 processed using Topspin 3.1 (Bruker, RRID:SCR_014227) and analysed using NMRFAM-
22 Sparky ((Lee et al., 2015), RRID:SCR_014228), the backbone assignment was aided using
23 MARS (Jung and Zweckstetter, 2004). The assigned backbone chemical shifts were used to
24 guide calculation of a structural model of the PDZ domain using POMONA/CS-RosettaCM
25 (Shen and Bax, 2015). Including a limited number of long-distance NOE restraints further

1 refined the model; these restraints were curated so that they were from amino acids 3 or more
2 residues apart as previously described (Raman et al., 2010).

3

4 *NMR dynamics characterization and binding experiments*

5 The dynamic properties of the DEPTOR PDZ protein were investigated using standard
6 Bruker ^{15}N T_1 , T_2 and $^{15}\text{N}[^1\text{H}]\text{NOE}$ [heteronuclearNOE] experiments. T_1 relaxation times were
7 calculated using delays of 10, 20, 40, 80, 160, 320, 640, 1280 and 2000 ms and T_2 relaxation
8 times with delays of 16.9, 33.8, 67.6, 101.4, 135.2, 169.0, 202.8 and 253.5 ms. Peak intensities
9 and curve fitting were calculated using Sparky.

10 To observe the interaction of DEPTOR PDZ with mTORC1, 32 μM of $^2\text{H},^{13}\text{C},^{15}\text{N}$
11 DEPTOR PDZ was added to 3.2 μM of the mTORC1 complex, with the excess PDZ shifting
12 the equilibrium towards a higher percentage of bound state. Here, binding is observed as a
13 residual effect in the unbound pool of PDZ and is manifested as line broadening in the $^1\text{H}-^{15}\text{N}$
14 HSQC experiment when compared to free DEPTOR PDZ only. The $^{15}\text{N}-^1\text{H}$ HSQC experiment
15 was used here instead of the $^1\text{H}-^{15}\text{N}$ BEST-TROSY to avoid potential solvent exchange bias.
16 Peaks heights were normalized to the signal of the C-terminal residue before the ratio
17 calculated. Peaks that had reduced relative intensity define the interaction surface for the PDZ
18 domain.

19 Structural differences between the DEPTOR PDZ domain and the DEPTOR linker-
20 PDZ construct were first identified by chemical shift perturbations in $^1\text{H}-^{15}\text{N}$ BEST-TROSY
21 experiments. In the absence of a complete linker-PDZ assignment each signal of the linker-
22 PDZ spectrum from a 114 μM sample was compared with that of the assigned PDZ domain
23 collected under the same conditions, giving a weighted chemical shift perturbation calculated
24 by:

$$\Delta\delta_{\text{total}} = \left(\delta H^2 + \left(\frac{\delta N}{5} \right)^2 \right)^{0.5}$$

with the smallest perturbation reported as minimal chemical shift perturbation map.

Both the mTOR-FRB domain and the DEPTOR linker construct were assigned in order to identify residues involved in the binding interaction. FRB assignments were obtained at 293K and transferred to the 278K spectra using a temperature titration. The backbone assignment of the 70 μM FRB sample (residues 2015-2111) was completed using 3D HNCO, HN(CA)CO and HNCACB, CBCA(CO)NH experimental pairs - all collected with 1024, 64 and 96 points in the proton, nitrogen and carbon dimensions respectively and with 20-40% non-uniform sampling (NUS). Data was processed using NMRPipe (Delaglio et al., 1995) including compressed sensing for data reconstruction (Kazimierczuk and Orekhov, 2011) and analysed as above for the PDZ data. A 140 μM $^{15}\text{N}/^{13}\text{C}$ DEPTOR linker (288-323) construct was assigned using the same experiment pairs as above, supplemented with HN(COCA)NNH and HN(CA)NNH experiments (2048, 64 and 80 points in the proton, nitrogen and carbon dimensions, and 25% NUS) to provide sequential N,N connectivities. Carbon-detect 3D experiments, in recent years established for the assignment of disordered proteins due to their superior resolution, allowed the completion of the sequential assignment including proline connectivities. Carbon-detect 3D experiment pairs (HCA)CON and (HCA)NCO were collected with up to 1024, 128 and 80 complex points in the carbonyl-carbon, nitrogen and indirect aliphatic-carbon dimensions respectively, whereas the CBCACON and CBCANCO pair was acquired with 1024, 72 and 80 points in carbonyl-carbon, nitrogen and indirect aliphatic-carbon dimensions.

Secondary chemical shift analysis to describe conformational preferences for the DEPTOR linker was based on a comparison of assigned backbone carbon α and β shifts with chemical shift values expected for a random coil protein with the same sequence and under the

1 same experimental conditions (pH and temperature) as calculated according to (Kjaergaard et
2 al., 2011; Kjaergaard and Poulsen, 2011; Schwarzingger et al., 2001). Random coil values were
3 subtracted from the experimental data to give $\Delta\sigma C\alpha$ and $\Delta\sigma C\beta$, with $\Delta\sigma C\alpha$ - $\Delta\sigma C\beta$ plotted
4 against residue number.

5 Binding of the DEPTOR linker to mTORC1 FRB was observed by ^1H - ^{15}N BEST-
6 TROSY NMR. FRB residues involved in the binding were identified by the addition of up to
7 220 μM of unlabelled linker-PDZ to 40 μM of ^{15}N labelled FRB. Similarly, DEPTOR linker
8 residues involved in binding were identified by the addition of up to 320 μM of unlabelled FRB
9 to a 40 μM ^{15}N labelled DEPTOR linker sample. Data from both titrations was analysed using
10 the above equation.

11

12 *HDX experiments*

13 Experiments followed suggested standards by the HDX-MS community (Masson et al.,
14 2019). For global exchange, a 5 μL solution of 5 μM DEPTOR (50 mM HEPES pH 8.0, 100
15 mM NaCl, 1 mM TCEP) was incubated for 3 s with 40 μL of ice-cold D_2O buffer of identical
16 composition (92.8% D_2O), for a final concentration of 74.24% D_2O . The reaction was
17 quenched using 20 μL of 2 M guanidinium chloride and 2.4% v/v formic acid, instantly shock-
18 frozen and stored at -80°C . For measurement of the incorporation of deuterium, samples were
19 thawed and injected onto an M-Class Acquity UPLC with HDX Manager technology (Waters)
20 kept at 0.1°C . Samples were digested on-line using an Enzymate BEH immobilized Pepsin
21 Column (Waters, 186007233) at 15°C for two min, with peptides being eluted onto an Acquity
22 UPLC BEH C18 column (Waters, 186002346), equilibrated in Buffer Pepsin-A (0.1% v/v
23 formic acid), using a 3-43% gradient of Pepsin-B buffer (0.1% v/v formic acid, 99.9%
24 acetonitrile) over 12 min. Data was collected on a Waters Synapt G2 Si using MS^e mode (Silva
25 et al., 2005), using an electrospray source (set at 3 kV), from 50 to 1800 m/z. Peptides were

1 identified from non-deuterated samples of DEPTOR in the independent replicates using
2 ProteinLynx Global Server (Waters, RRID:SCR_016664) against a library of DEPTOR and
3 porcine pepsin, and then imported into DynamX (Waters) software using the following criteria
4 for automated selection: Minimum Intensity 7500, Minimum sequence Length 6, Maximum
5 Sequence Length 30, Minimum Products 1, Minimum products per amino acid 0.11, Minimum
6 Consecutive products 1, Minimum Sum Intensity for products 500, Maximum MH⁺ error 5
7 ppm, and identification in all three non-deuterated files. 205 peptides were initially identified,
8 reduced to 179 after a manual quality control process (presence of overlapping peptides *etc.*)
9 was conducted.

10 The HDX binding study of FRB and DEPTOR was performed by preincubating 100
11 μ M FRB with 100 μ M DEPTOR in buffer (50 mM HEPES pH 8.0, 100 mM NaCl, 1 mM
12 TCEP) at room temperature for 1 h. An aliquot of 5 μ L was incubated with 45 μ L of D₂O
13 buffer at room temperature for 3, 30, 300 and 3000 s, the reaction was quenched and treated as
14 described, with the exception of using a 5-36% gradient of acetonitrile in 0.1% v/v formic acid
15 for elution from Acquity UPLC BEH C18 column. Data was collected from 300 to 2000 m/z,
16 and mass analysis was performed as described above. Deuterium incorporation was not
17 corrected for back-exchange and represents relative, rather than absolute changes in deuterium
18 levels. Changes in H/D amide exchange in any peptide may be due to a single amide or a
19 number of amides within that peptide. All time points in this study were prepared at the same
20 time and individual time points were acquired on the mass spectrometer on the same day.

21

22 *Surface Plasmon Resonance*

23

24 Twin-Strep-tagged wild-type and activated mTORC1 mutant A1459P were purified as

25 described above with the exception that no TEV-protease was added, and the protein was

1 eluted from the Strep-Trap HP columns using 10 mM desthiobiotin in 40 mL elution buffer
2 prior loading onto a 5 mL HiTrap Q column.

3 SPR was performed using a Biacore T200 using CM5-sensor chips (Cytiva). Both
4 reference control and analyte channels were equilibrated 50 mM Hepes, pH 7.5, 100 mM
5 NaCl, 1 mM TCEP. Twin-Strep-tagged mTOR was captured onto a Strep-Tactin XT (IBA
6 Lifesciences) coated surface prepared according to the supplied instructions. SPR runs were
7 performed with analytes injected for 120 s followed by a 300 s dissociation in a 1:2 dilution
8 series with initial concentrations of 20 μ M for DEPTOR PDZ (residues 305-409). After
9 reference and buffer signal correction, sensogram data were fitted using GraphPad Prism
10 (RRID:SCR_002798). The equilibrium response (R_{eq}) data were fitted to: a single site
11 interaction model to determine K_d :

$$12 \quad R_{eq} = \left(\frac{CR_{max}}{C+K_d} \right) + N_s C + B$$

13 where C is the analyte concentration and R_{max} is the maximum response at saturation, N_s is a
14 linear non-specific binding term and B is the background resonance; or a two-site model:

$$15 \quad R_{eq} = \left(\frac{CR_{max1}}{C+K_{d1}} \right) + \left(\frac{CR_{max2}}{C+K_{d2}} \right) + B$$

16 where R_{max1} and K_{d1} , and R_{max2} and K_{d2} are the maximum response and dissociation constants
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18

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7

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12 Review & Editing All authors.; Visualization M.H., A.B., J.W., R.L.W.; Supervision R.L.W.;
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14

15 **Competing interests**

16 The authors declare no competing interests.

17

18

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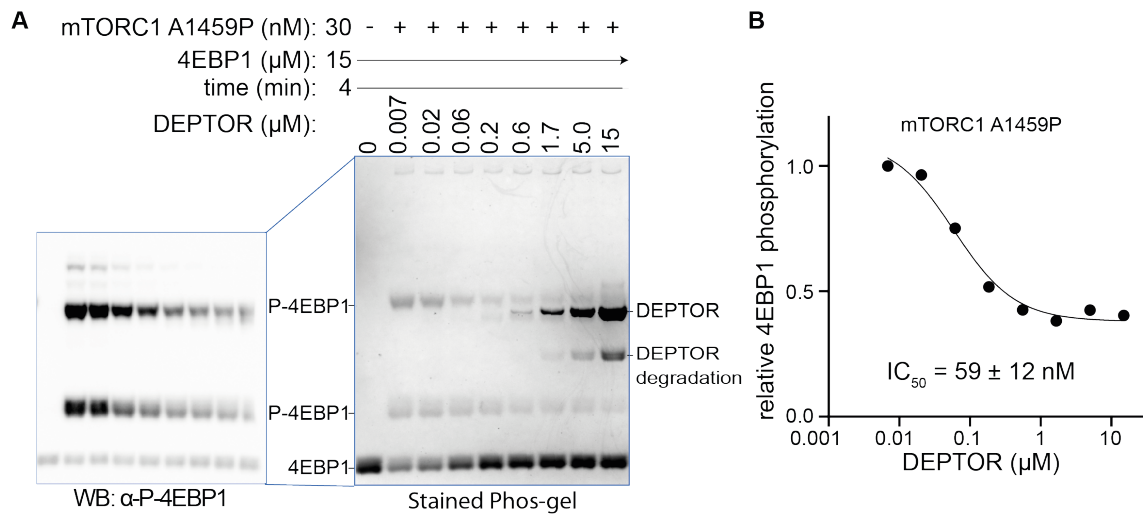
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1 Supplemental Figures



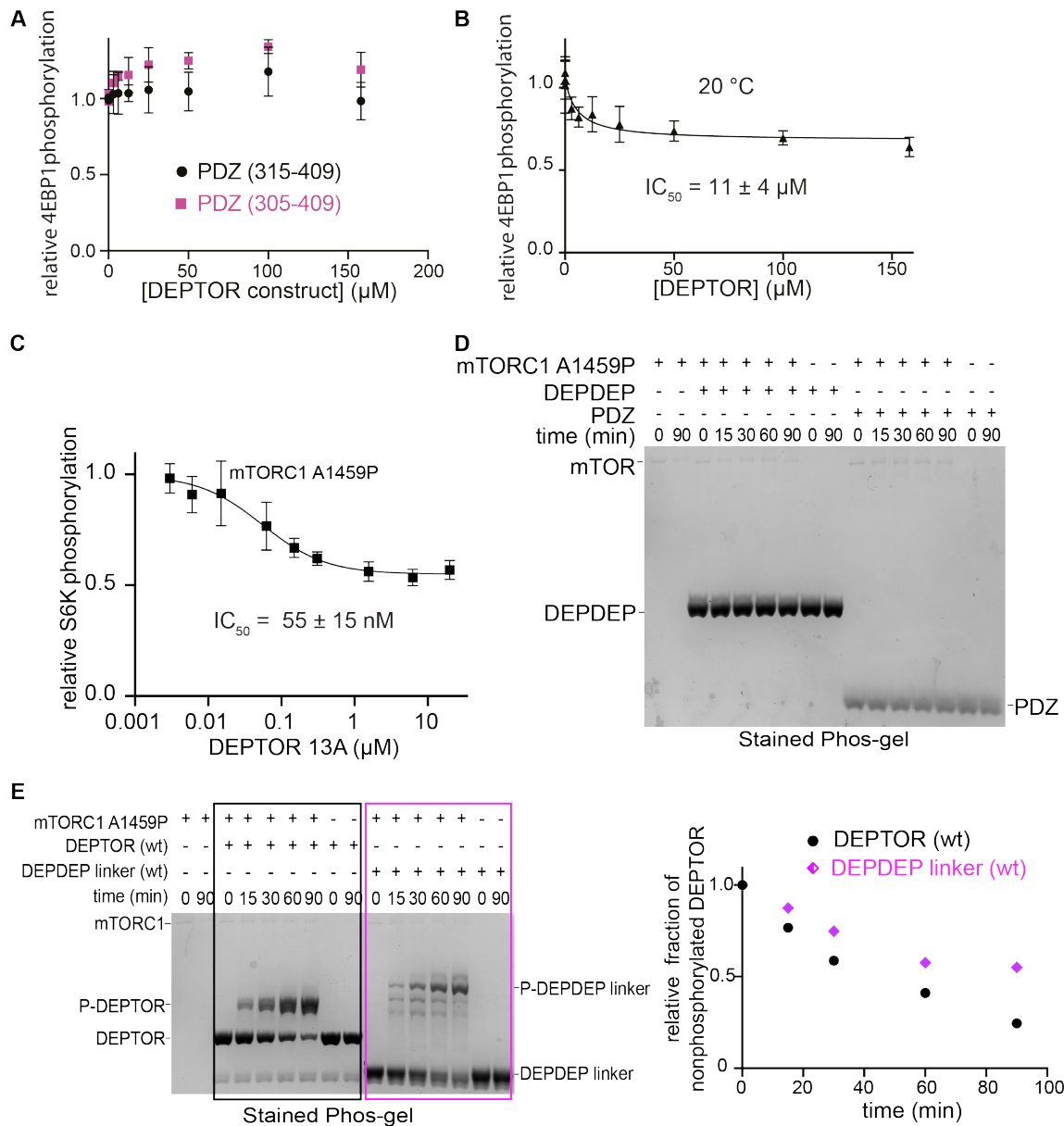
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3 **Figure 1-figure supplement 1. DEPTOR is a partial inhibitor of mTORC1, analysed by** 4 **Phos-tag SDS PAGE and western blot.**

5 (A) DEPTOR inhibition of mTORC1 cancer-associated mutant A1459P phosphorylating
6 4EBP1 measured by Phos-tag SDS PAGE, stained with InstantBlue. A second Phos-tag SDS
7 PAGE was used for a western blot analysis in order to identify all P-4EBP1 bands.

8 (B) The plot of mTORC1-A1459 activity measured by P-4EBP resolved by the Phos-tag gel
9 shown in (A). The plot is representative of three independent replicates. Intensity of all P-
10 4EBP1 bands in one lane was normalized to the P-4EBP1 intensity at the lowest DEPTOR
11 concentration. The IC_{50} for mTORC1 A1459P inhibition by DEPTOR was also tested via a
12 western blot experiment, resulting in a comparable value (Figure 6B).

13



1

2 **Figure 2-figure supplement 1. DEPTOR is a partial inhibitor of mTORC1, analysed by**
3 **Phos-tag SDS PAGE and western blot.**

4 (A) The PDZ construct (324-409) was extended at the N-terminus to encompass residues 305-
5 409 or 315-409 in order to obtain a more stable construct that could be used for assays at 30
6 °C. No inhibition of mTORC1 kinase activity was observed in a western blot experiment with
7 these two PDZ constructs at 30 °C.

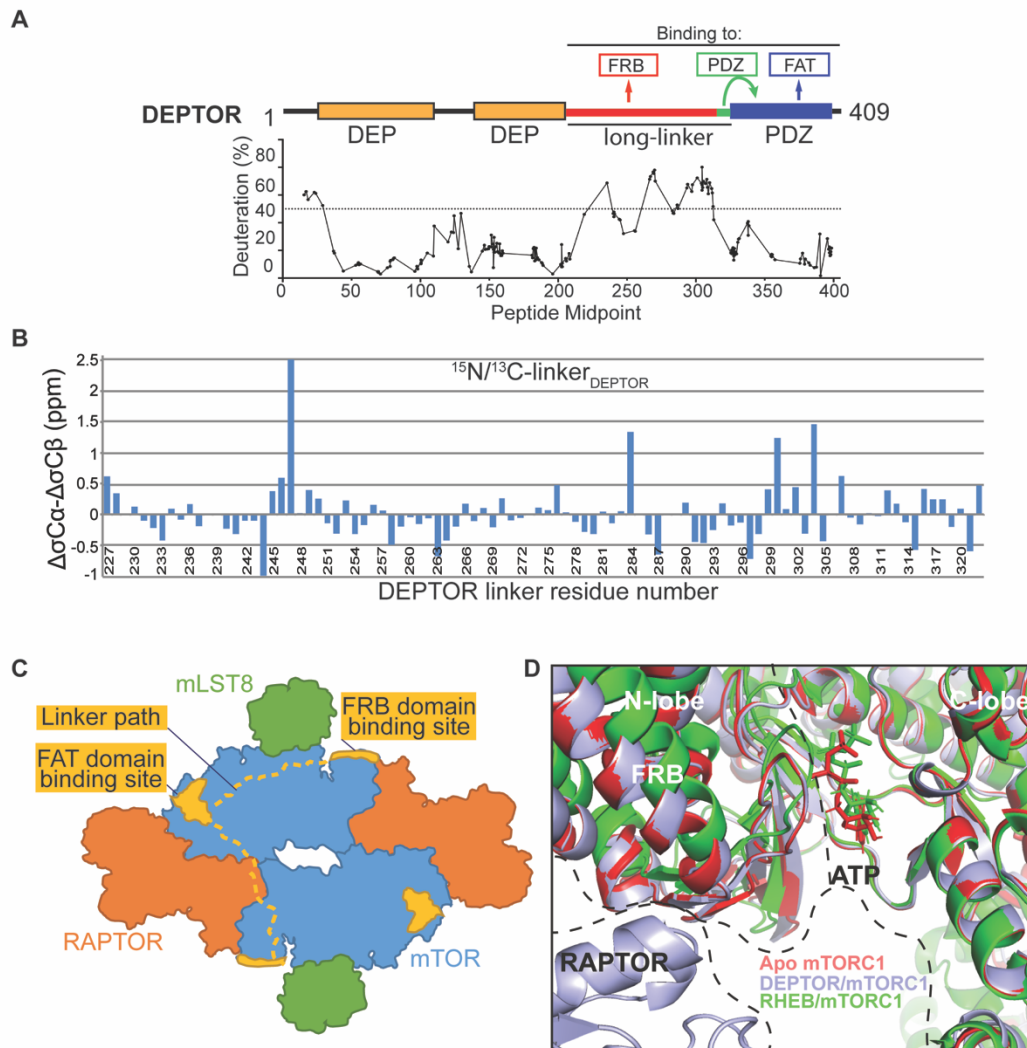
8 (B) DEPTOR inhibition of mTORC1 was tested at 20 °C (as compared with the results at 30
9 °C, as shown in Figure 2D) via western blot experiment to allow a direct comparison with the

1 inhibitory properties of temperature-sensitive DEPTOR PDZ domain. The data (mean \pm SD,
2 $n \geq 3$) were fit by a nonlinear regression to determine IC_{50} .

3 (C) DEPTOR 13A inhibition of the mTORC1-A1459P mutant phosphorylating GST-S6K³⁶⁷⁻
4 ⁴⁰⁴ peptide measured by western blots. Band intensities were normalized to the control (0 μ M
5 DEPTOR). The data (mean \pm SD, $n = 3$) were fitted.

6 (D) The tandem DEPDEP and the PDZ domain are not substrates of mTORC1. No
7 phosphorylation is observed in a Phos-tag SDS PAGE experiment.

8 (E) DEPTOR as an mTORC1 substrate was investigated via Phos-tag SDS PAGE. DEPDEP-
9 linker construct (residues 1-323) is phosphorylated slower than the full-length DEPTOR (WT),
10 suggesting that the PDZ domain helps successful interaction at the FRB and active site (one
11 representative of a triplicate experiment is shown).

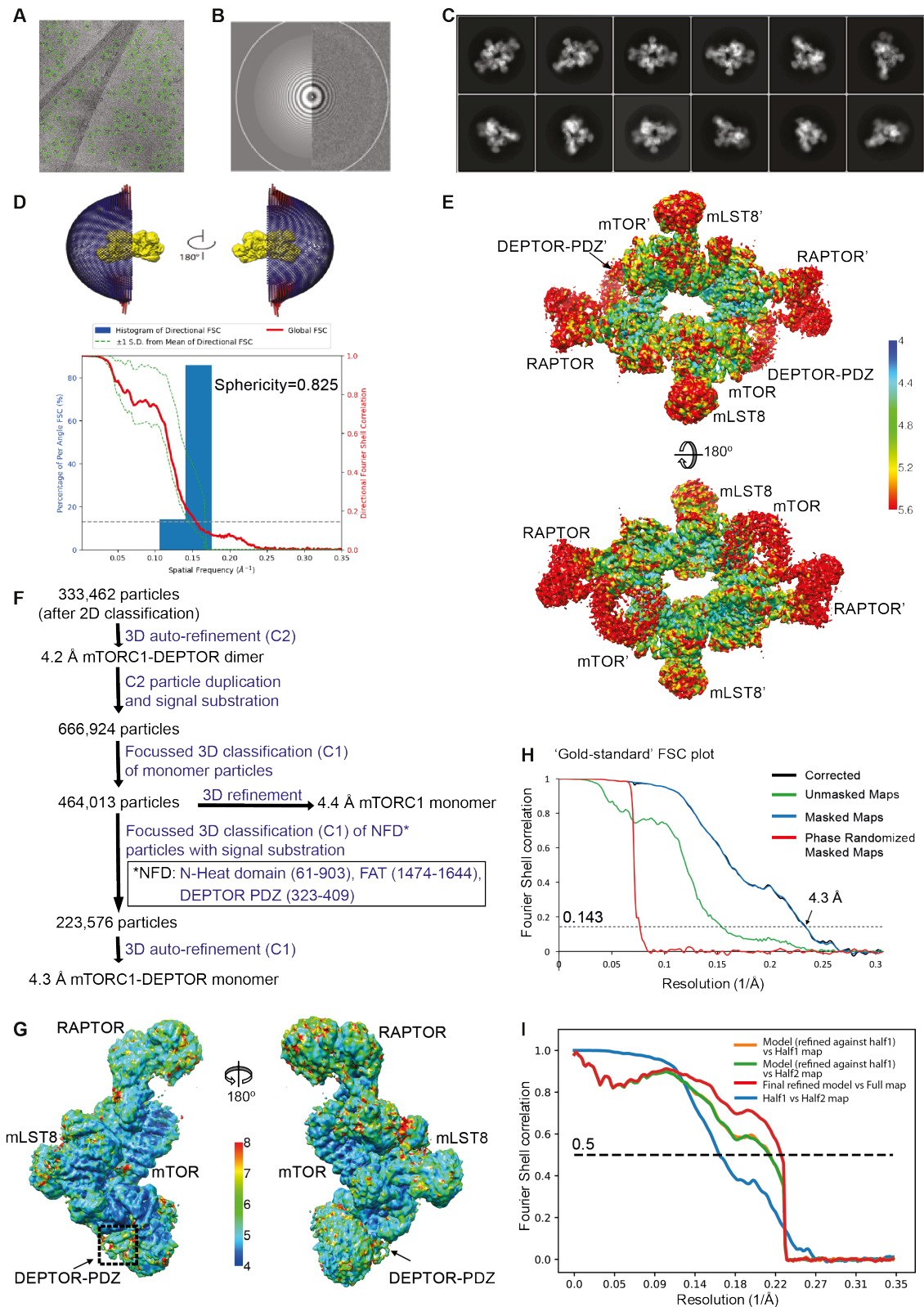


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2 **Figure 3-figure supplement 1. DEPTOR interaction with mTORC1.**

3 (A) Global HDX-MS result for DEPTOR as an indication of structured and flexible regions of
 4 DEPTOR. There were 179 reliable peptic peptides, with 94.9% coverage of DEPTOR and a
 5 redundancy of about 7.2. Areas of lower solvent exchange rate, suggestive of regions with
 6 likely secondary structure (Fowler et al., 2016), agreed with the predicted boundaries of the
 7 tandem DEP domains (36-119 and 145-219) and of the PDZ domain (330-409). There is also
 8 a region of lower solvent exchange rate observed in the inter-DEP-linker (residues 120-144,
 9 deuteration <50% in 3 s). In contrast, there is very rapid exchange for the long-linker (residues
 10 228-323) connecting the tandem DEP domains with the PDZ domain (>50% in 3 s), suggesting
 11 that this region is unstructured. See also Supplementary File 1.

- 1 **(B)** Secondary structure analysis of the DEPTOR linker construct (residues 228-323)
2 comparing the assigned linker shifts with those that would be expected in a peptide of random
3 coil conformation, revealing no regions of residual secondary structure in the unbound state.
4 In this plot residual structure would be indicated by stretches of the protein with values greater
5 than 1 for helical residues and greater than -1 for extended residues, which are not present for
6 the DEPTOR linker.
- 7 **(C)** A schematic illustrating two possible paths for the DEPTOR linker spanning the FRB and
8 FAT domain binding sites. As DEPTOR is inhibitory for the mTOR monomer
9 (Δ NmTOR/mLST8 construct), FRB and FAT domain binding can occur across the monomer.
10 This does not exclude an interaction across the dimer interface.
- 11 **(D)** Structure alignment on the C-lobe of free mTORC1 (pdb ID 6bcx), RHEB-bound
12 mTORC1 (pdb ID 6bcu), and DEPTOR-bound mTORC1 shows similarities of free and
13 DEPTOR-bound mTORC1 in the ATP binding site.



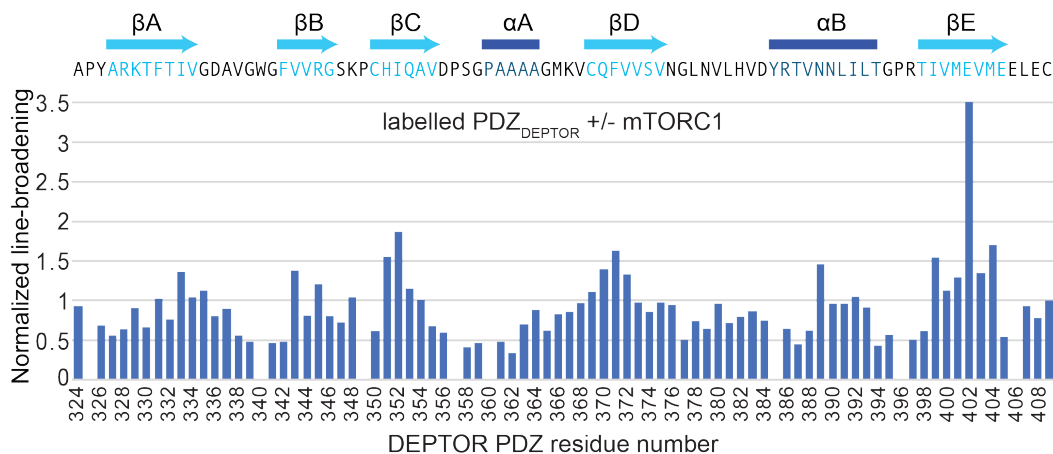
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2 **Figure 3-figure supplement 2. Cryo-EM data of the mTORC1/DEPTOR complex.**

3 (A) A representative cryo-EM micrograph of mTORC1-DEPTOR complex.

4 (B) Power spectrum of the image in (A) showing contrast transfer function (Thon rings)

- 1 (C) Some of the reference-free 2D classes are shown.
- 2 (D) The angular distribution (Euler angles) of the last 3D auto-refinement indicates some
3 overrepresented views (preferred orientation, red cylinders). Directional resolution of the final
4 3D reconstruction of EM map density of mTORC1-DEPTOR was calculated using the 3DFSC
5 server (Tan et al., 2017).
- 6 (E) The local resolution map of the 3D reconstruction is shown for the mTORC1 dimer together
7 with the resolution bar ranging from 4 Å to 5.6 Å (blue to red gradient).
- 8 (F) Flowchart of cryo-EM data processing. Details are described in methods.
- 9 (G) The local resolution map of the 3D reconstruction is shown for the mTORC1 monomer
10 together with the resolution bar ranging from 4 Å to 8 Å (blue to red gradient).
- 11 (H) The 'gold-standard' FSC curve for the B-factor sharpened post-processed reconstruction
12 suggests a final resolution of the mTORC1/DEPTOR complex refined as a monomer of 4.3
13 Å. The dashed line indicates the FSC value for 0.143.
- 14 (I) FSC curves of the final model versus the full map (red), of a same model refined in the
15 first independent half map versus the first half map (orange), and of the same model versus
16 the second independent half map, which was not used for refinement (green). FSC curve for
17 the first half map versus the second half map (blue) is also shown. The dashed line indicates
18 the FSC value for 0.5.
- 19

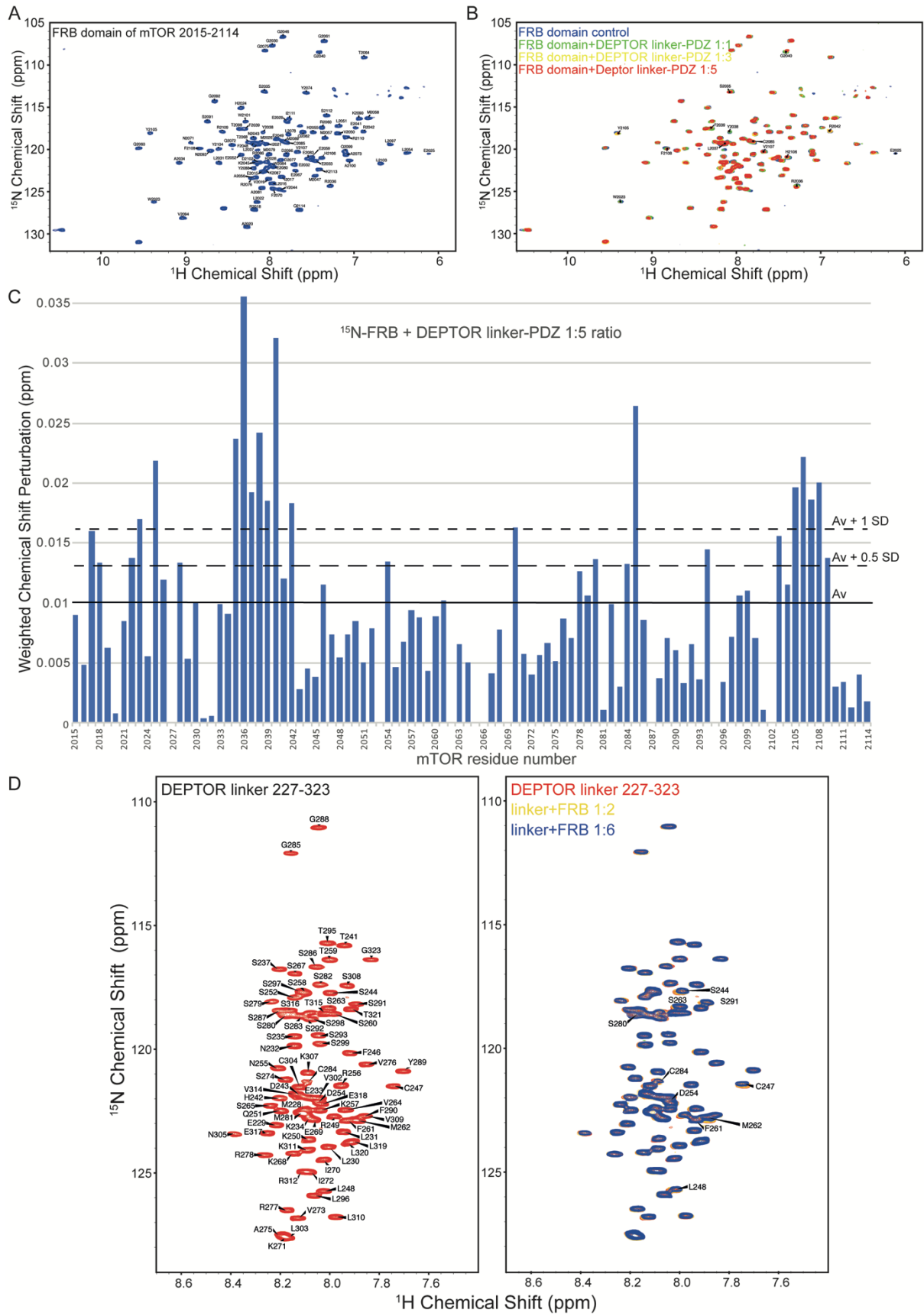


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2 **Figure 4-figure supplement 1. DEPTOR interaction with mTORC1.**

3 Normalized differential line-broadening analysis of deuterated DEPTOR PDZ domain binding
4 to mTORC1. Ratios of the $^2\text{H}^{13}\text{C}^{15}\text{N}$ PDZ (residues 324-409) peak intensity in the ^{15}N - ^1H
5 HSQC lower than 1 indicate line broadening and therefore the binding surface of mTORC1
6 interaction with isolated DEPTOR PDZ domain (residues 324-409). Measured peak intensity
7 was normalized to the peak of the C-terminal residue.

8



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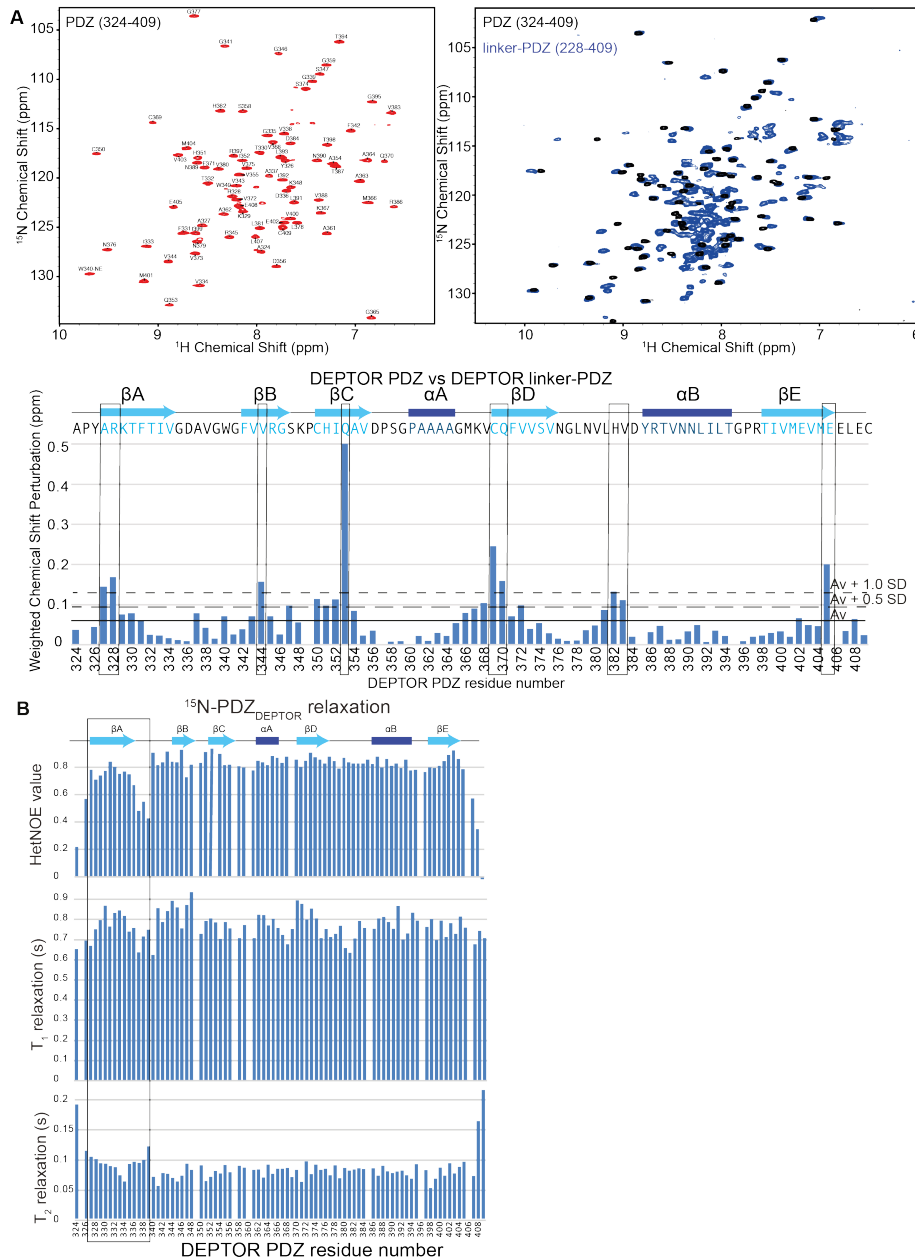
1 **Figure 5-figure supplement 1. Analysis of the mTOR FRB domain and DEPTOR linker**
2 **interaction by NMR.**

3 (A) The assigned ^1H - ^{15}N BEST-TROSY spectrum of $^{13}\text{C}/^{15}\text{N}$ mTOR-FRB (residues 2015-
4 2114).

5 (B) Overlaid spectra of ^{15}N labelled FRB with increasing concentrations of DEPTOR linker-
6 PDZ (residues 228-409) (FRB alone, blue; FRB:linker-PDZ= 1:1, green; FRB:linker-PDZ =
7 1:3, yellow; FRB:linker-PDZ = 1:5, red).

8 (C) The weighted chemical shift perturbation map showing residues of the FRB that bind
9 DEPTOR linker-PDZ. These data were used to create the heat map in Figure 5A.

10 (D) The assigned ^1H - ^{15}N BEST-TROSY spectrum of DEPTOR-linker (residues 228-323) and
11 overlaid spectra of ^{15}N labelled DEPTOR linker with increasing concentrations of mTOR FRB
12 (DEPTOR linker alone, red; DEPTOR linker:FRB=1:2, yellow; DEPTOR linker:FRB=1:6,
13 blue). A subset of perturbed peaks has been labelled and the spectrum of 1:6 ratio of DEPTOR
14 linker to FRB was used to create the weighted chemical shift map shown in Figure 5B.

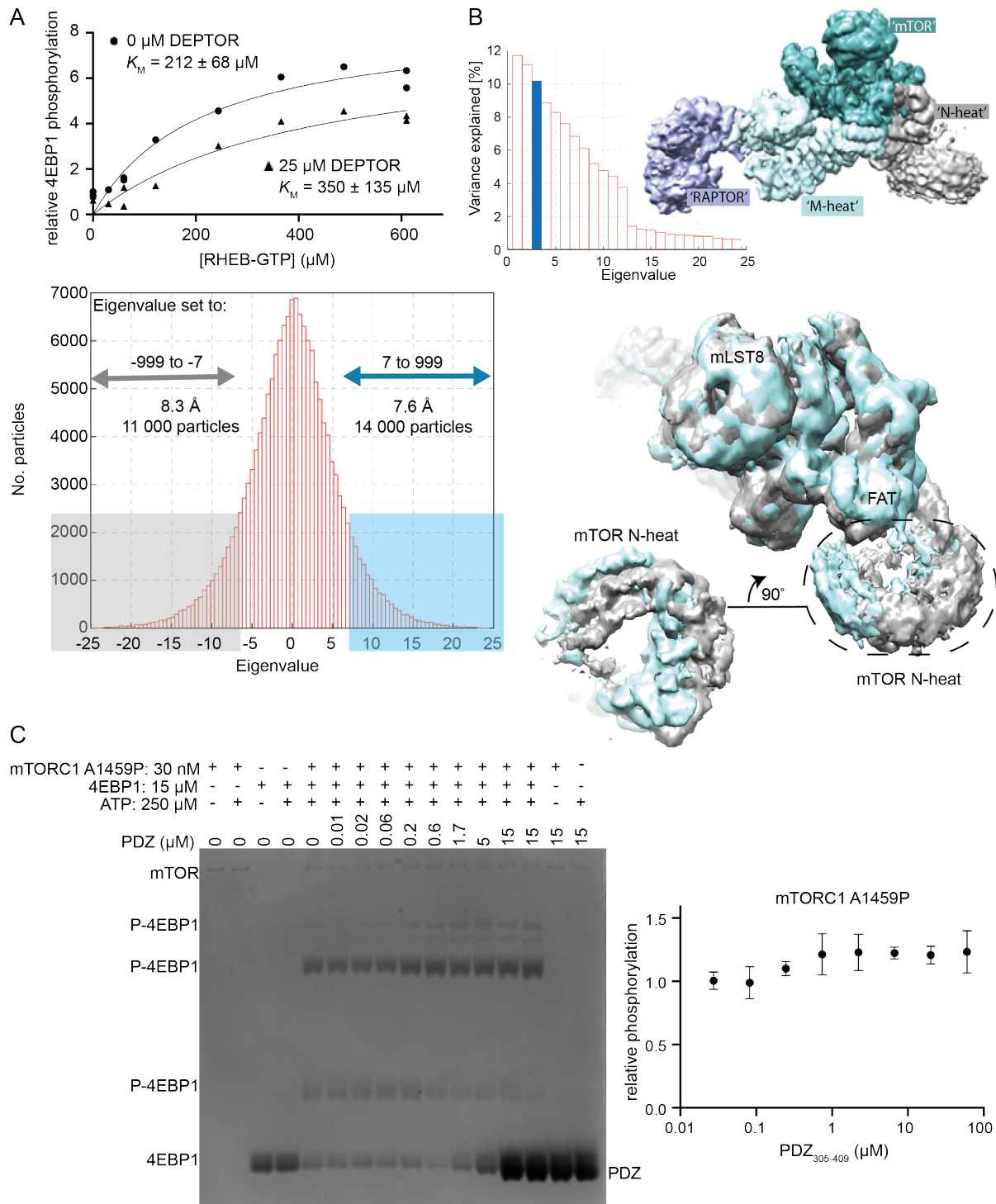


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2 **Figure 5-figure supplement 2. DEPTOR PDZ domain is greatly stabilized by its**
 3 **interaction with a short stretch of DEPTOR linker.**

4 (A) NMR analysis of PDZ and linker-PDZ of DEPTOR. Top left: Assigned ^{15}N - ^1H BEST-
 5 TROSY spectrum of the PDZ domain (residues 324-409) of DEPTOR. TOP right: Comparison
 6 of PDZ and linker-PDZ (residues 228-409) ^{15}N - ^1H BEST-TROSY spectra. Bottom: A minimal
 7 chemical shift perturbation map showing the shortest distance between the assigned peaks of
 8 the PDZ spectrum and the unassigned peaks of the linker-PDZ of DEPTOR. Relatively large

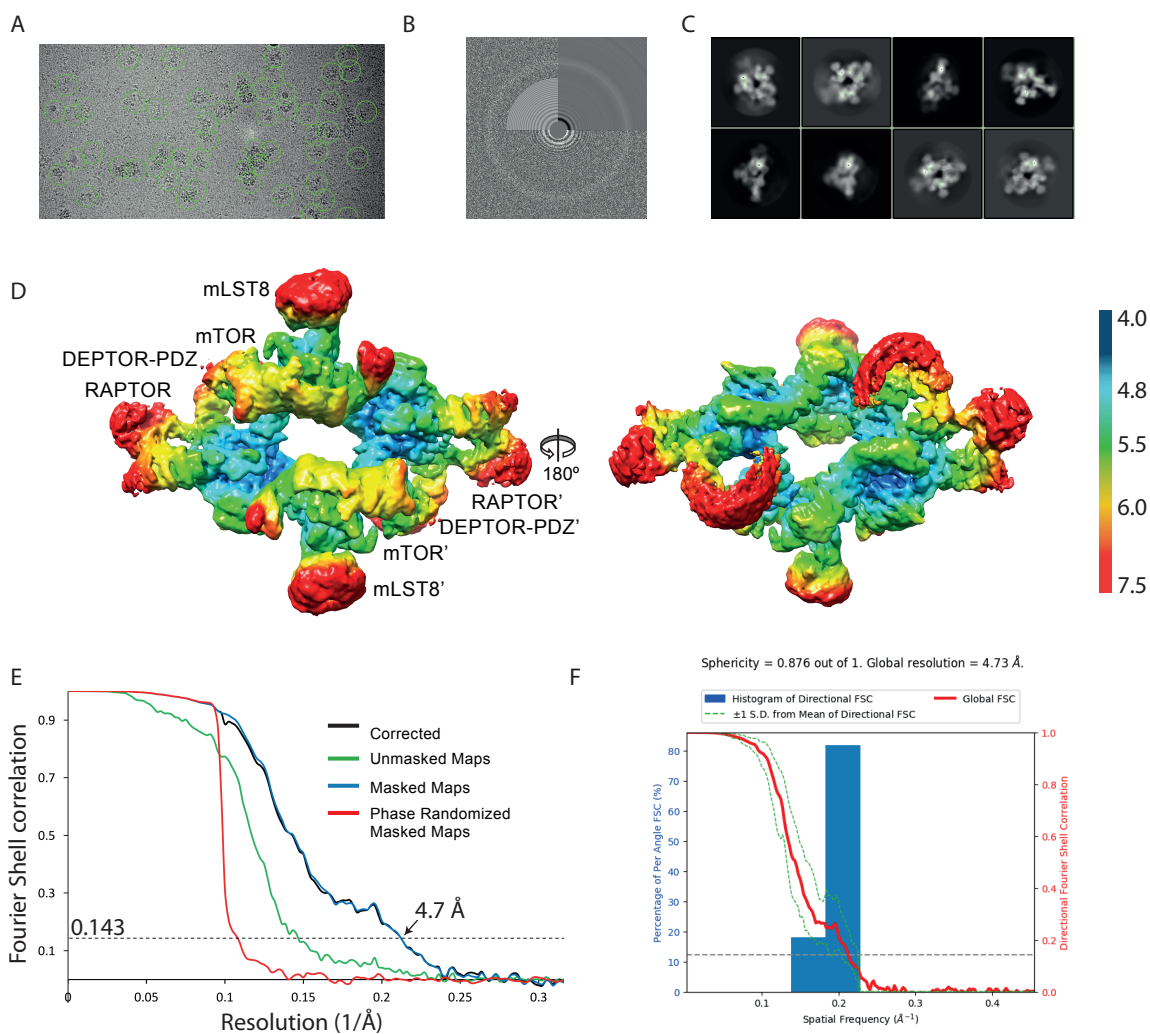
1 peak perturbation differences are attributed to an interaction of the linker with the folded PDZ
2 domain and are used to make the heat map in Figure 5D.
3 **(B)** ^{15}N dynamics analysis of $^{15}\text{N}/^2\text{H}$ labelled DEPTOR PDZ domain. Top: ^{15}N - ^1H
4 Heteronuclear NOE, middle: T_1 – longitudinal, and bottom: T_2 – transverse relaxation times
5 for the PDZ construct (324-409) collected at 700 MHz. The N-terminal region of the PDZ
6 shows increased flexibility.



1 fraction of the control (no DEPTOR/ no RHEB) vs. activated/ inhibited. One representative of
2 three replicates is shown.

3 **(B)** For multibody refinement, the wild-type mTORC1/ DEPTOR complex was split into four
4 bodies. The refinement revealed that about 33% of variation in rotation and translation is
5 explained by the first three eigenvectors. The first two eigenvectors correspond to a twisting
6 motion in RAPTOR and a slight tilt in mLST8, respectively, while the third shows a rolling
7 motion in the N-heat domain. Histograms of all amplitudes were monomodal, implying
8 continuous motions. We selected and refined ~11 000 particles for which the amplitude along
9 the third eigenvector is ≤ -7 (yielding the density shown in grey) and ~ 14 000 particles with
10 the amplitude > 7 (shown in cyan). These refinements yielded maps with overall resolutions of
11 8.3 Å and 7.6 Å, respectively, and show the motion in the N-heat and FAT regions.

12 **(C)** Short-linker PDZ does not inhibit mTORC1 A1459P, as analysed by Phos-gels. Despite
13 the isolated PDZ domain (short-linker PDZ construct 305-409) binding tighter to mTORC1
14 A1459P than wild-type mTORC1, no inhibition of the mutant was observed. Quantification of
15 western blots of phosphorylated 4EBP1 plotted as a fraction of the control (0 μ M PDZ) vs.
16 PDZ (mean \pm SD, n = 3).



1

2 **Figure 6-figure supplement 2. Cryo-EM data of the mTORC1 A1459P/DEPTOR**
3 **complex.**

4 (A) A representative cryo-EM micrograph of mTORC1 A1459P-DEPTOR complex.

5 (B) Power spectrum of the image in (A) showing contrast transfer function rings (Thon rings).

6 (C) Some of the 2D classes are shown.

7 (D) The local resolution map of the 3D reconstruction is shown for the mTORC1 dimer
8 together with the resolution bar ranging from 4 Å to 7.5 Å (blue to red gradient).

9 (E) The 'gold-standard' FSC curve for the B-factor sharpened post-processed reconstruction
10 suggests a final resolution of the mTORC1 A1459P/DEPTOR complex of 4.7 Å.

- 1 (F) Directional resolution of the final 3D reconstruction was calculated using the 3DFSC
- 2 server (Tan et al., 2017).

1 **Supplementary File 1. Global HDX-MS measurements for the full-length, human**

2 **DEPTOR**

3
4 **Supplementary File 2. Summary of HDX-MS results for DEPTOR in the presence and**
5 **absence of the mTOR-FRB domain, related to Figure 5C.**

6
7 **Table 1. Cryo-EM data collection and processing of mTORC1 in complex with DEPTOR.**

| Data collection | | |
|---|-------------------|----------------------|
| Protein details | mTORC1 WT/DEPTOR | mTORC1 A1459P/DEPTOR |
| Microscope | Titan Krios (FEI) | Titan Krios (FEI) |
| Voltage (kV) | 300 | 300 |
| Detector | Gatan K2 Summit | Gatan K3 |
| Pixel size (Å) | 1.43 | 1.1 |
| Defocus range (µm) | -1.6 to -3.2 | -1.4 to -3.0 |
| Movies | 2370 | 4759 |
| Frames/movie | 20 | 50 |
| Exposure rate (e ⁻ / Å ² / s) | 2.5 | 22.4 |
| Total dose (e ⁻ / Å ²) | 40 | 56 |
| Number of particles | 491,404 | 97,314 |
| Energy filter slit width (eV) | 20 | 20 |
| Model composition | | |
| Non-hydrogen atoms (monomer) | 28917 | 28784 |
| Protein residues | 3640 | 3598 |
| Ligands/ ions | - | - |
| Density Refinement | | |
| Resolution (Å) | 4.4 | 4.7 |
| Sharpening B-factor (Å) | 283.5 | 145.7 |
| Model refinement | | |
| Root-mean-square deviation | | |
| Bond lengths (Å) | 0.008 | 0.0091 |
| Bond angles (°) | 1.23 | 1.88 |
| <i>Validation</i> | | |
| Molprobit score | 1.9 | 1.8 |
| Clashscore, all atoms | 2.3 | 1.0 |
| Favored rotamers (%) | 89.4 | 86.4 |
| Ramachandran plot (%) | | |
| Favored | 92.6 | 91.3 |
| Allowed | 6.65 | 8.2 |
| Outliers | 0.75 | 0.51 |

8