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Massachusetts Institute of Technology

Supplementary Materials for

Structural basis for the docking of mTORC1 on the lysosomal surface

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MATERIALS AND METHODS

Construction of protein expression plasmids

To support expression of multiprotein complexes, such as the Rag GTPases and Ragulator, we upgraded our custom-made bacterial expression line called pFloat *(39, 40)* into a two-tier assembly-expression system. First, we minimized the plasmid size by removing all fossil sequences (*f1 ori*, split *tetR*, *mbeC*), some of which remain from the original pBR322 plasmid *(41)*. Second, we incorporated stronger and shorter gene expression elements from the open source Registry of Standard Biological Parts (iGEM/MIT). For proteins that require lower temperatures for expression, we assembled an alternative backbone containing the cold shock protein (*cspA*) promoter *(42)*. Third, to facilitate PCR amplification and transfer of complete gene expression cassettes, we flanked them with 15-nt Prefix and Suffix sequences *(43)*. This resulted in the construction of tier-one plasmids, pFloat.T7 and pFloat.cspA, which serve as “entry” vectors for cloning genes with desired tags under the control of T7 or cspA promoters. Lastly, we constructed a tier-two plasmid, pFloat.assemble that serves as a destination vector for assembling multiple gene cassettes derived from tier-one plasmids. This cloning strategy relies on sequence homology between the ends of individual gene cassette fragments and allows for the construction of multigene expression plasmids in a single reaction *(43)*.

For the expression of more challenging proteins, such as Raptor, we put together a compact vector for use in mammalian cells called pDarmo. Similarly to the upgraded pFloat line, pDarmo was also designed with a tier hierarchy in mind. We tested many published expression cassettes in search of the highest yields of intracellular proteins in HEK293 cells. All vectors that included a CMV promoter, supported by a number of extra enhancing and stabilizing sequences, performed best in this trial with only minimal differences between the top scorers. One of the best variants, which we called “CMVT” *(44)*, was chosen as our primary vector due to its excellent GC content (52%) for PCR. pDarmo.CMVT can be used directly for protein expression via transient transfection, or indirectly through a BacMam baculovirus system *(45)*.

Both pFloat and pDarmo vectors carry a ccdB death cassette which significantly improves selection of positive clones, but requires specialized ccdB-resistant *E. coli* strains for

propagation, *e.g.* DB3.1 or ccdB-survival *(46)*. The pFloat and pDarmo lines are available from Addgene (see Table S1 for accession codes).

Protein expression and purification

Ragulator from bacteria. Coding sequences of all five human Ragulator genes were amplified from cDNA, and each cloned into a pFloat.T7 entry vector as follows: (1) GST-3C-p18(6-161), (2) p14(1-125), (3) MP1(1-124)-TEV-TwinStrep, (4) His6-3C-c7orf59(2-99), (5) HBXIP(1-91). The resulting gene expression cassettes were amplified with PCR and cloned into pFloat.assemble using MODAL overlaps *(43)*. All cloning steps were performed with seamless assembly methods *(47)*. The assembled multi-gene Ragulator construct was transformed into an engineered *E. coli* strain LOBSTR(DE3) *(48)*, carrying a pRARE2-pLysS plasmid (Novagen), and grown at 37°C in terrific broth (TB) medium. Protein expression was induced at OD_{600} 3.0 with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG), and the growth continued for another 20 h at 18 °C. The cells were harvested and resuspended in lysis buffer containing 50 mM HEPES, pH 7.5, 100 mM NaCl, 0.1% Tween-20, 1 mM tris(2-carboxyethyl)phosphine (TCEP), 0.5 mM phenylmethanesulfonyl fluoride (PMSF), protease inhibitor cocktail (Roche #11873580001, 1 tablet per 50 mL), 0.25 mg/ml lysozyme (Sigma Aldrich #L1667) and Universal Nuclease (0.02 µL per mL of lysate, Pierce #88700). Resuspended cells were lysed in an LM10 microfluidizer (Microfluidics) under 15,000 PSI air pressure, and the processed cell lysate was cleared by centrifugation at 35,000×g (JLA-16.250, Beckman Coulter) and 0.45 µM filtration. Clarified lysates were supplemented with 20 mM imidazole prior to incubation with Ni-NTA metal affinity agarose resin (QIAGEN #30210) pre-equilibrated with the binding buffer containing 50 mM HEPES, pH 7.5, 100 mM NaCl, 20 mM imidazole, 0.5 mM TCEP, 0.5 mM PMSF, 0.1% Tween-20. The affinity beads were washed with 50 column volumes (CV) of binding buffer, followed by 50 CV of the binding buffer supplemented with extra 200 mM NaCl, 20 mM $MgCl_2$ and 5 mM ATP. The Ragulator complex was released from beads after a 16 h incubation with HRV3C protease. Further purification was performed by size-exclusion chromatography (SEC) using HiLoad Superdex 200 16/600 columns (GE Healthcare) equilibrated in 25 mM HEPES, pH 7.5, 100 mM NaCl, 0.5 mM TCEP. Pure protein fractions were concentrated by centrifugal ultrafiltration, and their resulting concentration estimated by UV absorption at 280 nm.

Rag GTPases from bacteria. Coding sequences of RagA and RagC were optimized for expression in *E. coli*, and cloned into pFloat.cspA entry vectors as follows: (1) RagA(1-313), (2) His6-3C-RagC(2-399, S75N, T90N). The Follicular lymphoma mutations *(22)* were introduced using seamless cloning methods *(47)*, and the resulting gene expression cassettes assembled into a single pFloat.assemble plasmid (similarly to Ragulator).
The assembled multi-gene Rag GTPases construct was transformed into LOBSTR(DE3) cells *(48)*, carrying a pRARE2-pLysS plasmid (Novagen), and grown at 37°C in TB. Protein expression was induced at OD600 3.0 with 0.5 mM IPTG following a 45 min cold-shock in an ice bath, after which the growth was continued for another 48 h at 15 °C. The cells were harvested and resuspended in lysis buffer containing 50 mM HEPES, pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 0.05% Triton X-100, 1 mM TCEP, 0.5 mM PMSF, protease inhibitor cocktail (Roche #11873580001, 1 tablet per 50 mL), 0.25 mg/ml lysozyme (Sigma Aldrich #L1667) and Universal Nuclease (0.02 µL per mL of lysate, Pierce #88700). Resuspended cells were lysed in an LM10 microfluidizer (Microfluidics) under 15,000 PSI air pressure, and the processed cell lysate was cleared by centrifugation at 35,000×g (JLA-16.250, Beckman Coulter) and 0.45 µM filtration. Clarified lysates were supplemented with 20 mM imidazole prior to incubation with Ni-NTA metal affinity agarose resin (QIAGEN #30210) pre-equilibrated with the binding buffer containing 50 mM HEPES, pH 7.5, 100 mM NaCl, 20 mM imidazole, 0.5 mM TCEP, 0.5 mM PMSF, 2 mM $MgCl_2$, 0.01% Triton X-100. The affinity beads were washed with 50 CV of binding buffer, followed by 50 CV of the binding buffer supplemented with extra 200 mM NaCl, 18 mM $MgCl_2$ and 5 mM ATP. The Rag complex was released from beads after a 16 h incubation with HRV3C protease. Further purification was performed by SEC using HiLoad Superdex 200 16/600 columns (GE Healthcare) equilibrated in 25 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 0.5 mM TCEP. Pure protein fractions were concentrated by centrifugal ultrafiltration, and their resulting concentration estimated by UV absorption at 280 nm (corrected for the bound nucleotides).

Rag GTPases from mammalian cells. Coding sequences of RagA and RagC were cloned into the pRK5 vector as follows: (1) Flag-RagA(1-313), (2) HA-RagC(2-399). Mutations were introduced using seamless cloning methods *(47)*. Freestyle293 cells (Gibco) grown in suspension at 37 °C in a serum-free Freestyle293 medium (Gibco), were seeded at the density of 1 × 10^6 per mL, and co-transfected (polyethylenimine, PEI) with a pair of plasmids coding for RagA and RagC. The cells were harvested 72 h post-

transfection, and resuspended in lysis buffer containing 50 mM HEPES, pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 1% Triton X-100, 1 mM TCEP, 0.5 mM PMSF, protease inhibitor cocktail (Roche #11873580001, 1 tablet per 50 mL) and Universal Nuclease (0.02 µL per mL of lysate, Pierce #88700). Resuspended cells were allowed to lyse by mild detergent treatment (1% Triton-X 100) whilst stirring at 4°C for 30 min, and the resulting cell lysate was cleared by centrifugation at 50,000×g (JA 25.50, Beckman Coulter). Clarified lysates were incubated with anti-Flag affinity resin (Sigma #A2220) pre-equilibrated with the binding buffer containing 50 mM HEPES, pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 0.1% Tween-20, 0.5 mM TCEP, 0.5 mM PMSF. The affinity beads were washed with 50 CV of binding buffer, followed by 50 CV of the binding buffer supplemented with extra 200 mM NaCl, 18 mM $MgCl_2$ and 5 mM ATP. The Rag complex was released from beads with 10 CV of the binding buffer supplemented with 0.5 mg/mL 3xFlag peptide. Further purification was performed by SEC using Superdex 200 10/300 columns (GE Healthcare) equilibrated in 25 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 0.2 mM TCEP. Pure protein fractions were concentrated by centrifugal ultrafiltration, and their resulting concentration estimated by UV absorption at 280 nm (corrected for the bound nucleotides).

Raptor from mammalian cells. The coding sequence for Raptor was cloned into a pDarmo.CMVT entry vector as follows: Raptor(1-1335)-3C-Flag-TwinStrep. The expression cassette was recombined into a baculovirus genome (EmBacVSVG *(49)*) using the Bac-to-Bac protocol *(50)*, and the resulting bacmid was transfected into *Spodoptera frugiperda* (Sf9) insect cells to produce recombinant baculoviruses. Sf9 cells were cultured in Sf-900 III SFM (Gibco) medium at 27 °C, and the virus was amplified twice to support large-scale protein expression. Expi293 cells (Gibco) grown in suspension at 37 °C in a serum-free Expi293 medium (Gibco), were infected with Raptor baculoviruses (15% v/v) after reaching density of 4×10^6 per mL. After 24 h from infection, the culture was supplemented with 4 mM valproic acid to increase protein expression, and the growth was continued at 30 °C for another 48 h. The cells were harvested and resuspended in lysis buffer containing 50 mM HEPES, pH 8.0, 500 mM NaCl, 0.3% CHAPS, 10% glycerol, 1 mM TCEP, 1 mM EDTA, 0.5 mM PMSF, protease inhibitor cocktail (Roche #11873580001, 1 tablet per 50 mL) and Universal Nuclease (0.02 µL per mL of lysate, Pierce #88700). Resuspended cells were allowed to lyse by mild detergent treatment (0.3% CHAPS) whilst stirring at 4°C for 30 min, and the resulting cell lysate was cleared by centrifugation at 165,000×g (Ti45, Beckman Coulter). Clarified lysates were incubated

with StrepTactin XT affinity resin (IBA #2-4010) pre-equilibrated with the binding buffer containing 50 mM HEPES, pH 8.0, 500 mM NaCl, 0.3% CHAPS, 0.5 mM TCEP, 0.5 mM PMSF. The affinity beads were washed with 50 CV of binding buffer, followed by 50 CV of the binding buffer supplemented with extra 20 mM $MgCl_2$ and 5 mM ATP. Raptor was released from beads with 10 CV of the binding buffer supplemented with 100 mM biotin. Further purification was performed by SEC using Superose 6 10/300 columns (GE Healthcare) equilibrated in 25 mM HEPES, pH 8.0, 100 mM NaCl, 0.3% CHAPS, 1 mM TCEP. Pure protein fractions were concentrated by centrifugal ultrafiltration, and their resulting concentration estimated by UV absorption at 280 nm.

Protein complex reconstitution

The Raptor-Rag-Ragulator complex was reconstituted by mixing 2.6 nmol of Raptor, 2.7 nmol of the Rag GTPase heterodimer, and 2.7 nmol of Ragulator in a 500 µL volume in the presence of 0.25 mM GTP and 1 mM GDP. After a 16 h of incubation at 4°C, the protein mixture was injected on two Superose 6 10/300 columns (GE Healthcare) connected in tandem, and pre-equilibrated with the running buffer: 25 mM HEPES, pH 7.5, 100 mM NaCl, 0.5 mM TCEP, 0.3% CHAPS, 0.5 mM $MgCl_2$. Fractions containing the full Raptor-Rag-Ragulator complex were concentrated by centrifugal ultrafiltration, and their resulting concentration estimated by UV absorption at 280 nm (corrected for the bound nucleotides).

Cryogenic transmission electron microscopy

Cryo-EM specimens were prepared with a Vitrobot Mark IV (Thermo Fisher Scientific). 3 µL of 8 µM Raptor-Rag-Ragulator complex was applied to glow-discharged gold 300 square mesh Quantifoil R 1.2/1.3 holey carbon grids (Quantifoil). The grids were then blotted from both sides for 3 s at 90% humidity, before being plunge-frozen in liquid ethane. The protein complexes were imaged on a Titan Krios electron microscope (Thermo Fisher Scientific) operated at 300 kV. SerialEM *(51)* was used to collect micrographs in super-resolution counting mode on a Gatan K2 Summit direct electron detector at the end of a Gatan Quantum energy filter (slit width 20 eV). The total dose on the specimen was 42 e^- per $Å^2$ fractionated over 40 frames with a calibrated pixel size of 0.529 Å for the super-resolution micrographs.

Image processing – Raptor-Rag-Ragulator

Data processing workflow is illustrated in Fig. S7. A total of 5,340 super-resolution movies were corrected for drift (unbinned) using Relion's MotionCor2 implementation *(52, 53)*, and contrast transfer function (CTF) parameters were determined using GCTF *(54)*. The micrographs were denoised with JANNI *(55)*, and an initial subset of 1,000 particles was selected manually, and used to train deep-learning particle picking algorithms, Topaz or crYOLO *(23, 24)*. In our hands, these were the only pickers able to successfully select particles in holes and not on carbon, which was completely saturated with aggregated protein. To obtain an initial set of top quality particles, we used high stringency settings, which resulted in a total of 45,381 particles. After extraction and downscaling, these particles were used in reference-free 2D classifications in Relion *(56)*. The resulting 2D classes allowed us to generate an initial 3D reconstruction *de novo*, which we then used as a reference for 3D classifications. At this point, 2D projections of the best 3D class that represented a fully assembled complex were made, and after applying a low-pass filter to 40 Å, these projections were used as references for initial 2D classifications of a larger set of particles (1,293,759) that was picked with a lower stringency setting. Three rounds of reference-free 2D classifications were used to remove incorrectly selected particles and those of incomplete complexes. The remaining 302,760 particles were re-classified in 3D, and then used for initial refinement and reconstruction that led to a map with a resolution of 3.9 Å. Iterative cycles of beam-tilt refinement, per-particle defocus refinement and 3D refinement improved the map to 3.4 Å. Further processing of re-extracted movies using particle beam-induced motion correction and radiation-damage weighting *(57)* resulted in a polished data set that was then subjected to 3D classification without alignment. Four of the eight final 3D classes were combined and the resulting 112,037 particles were used for further non-uniform refinement in cryoSPARC *(58)*. The final map obtained from these particles was sharpened with a B-factor of -75 $Å^2$ and estimated at 3.18 Å resolution, according to 'gold standard' Fourier shell correlation (FSC) of 0.143 (Fig. S8). The directional resolution of the final map was measured with the 3DFSC server *(59)*. Local resolution was estimated in cryoSPARC *(58)* to extend from 2.3 to 4.2 Å (Fig. S9). Variability analysis was performed in cryoSPARC *(58).*

Image processing – Rag-Ragulator

Given the weak binding affinity between Raptor, Rag GTPases and Ragulator, and the fact that we used an excess of Rag GTPases and Ragulator in our reconstitution reaction with Raptor, we expected that at least a small percentage of the particles in our

micrographs would correspond to the sub-complex of Raptor-Rag and Rag-Ragulator. Indeed, after extensive 3D classifications, we found both of those 3D classes in our particle sets. While the Raptor-Rag was very difficult to cleanly separate from the full Raptor-Rag-Ragulator supercomplex, we managed to isolate 14,186 particles of the Rag-Ragulator sub-complex that eventually produced an 8.9 Å reconstruction. First, we erased the Raptor density from our Raptor-Rag-Ragulator supercomplex map in UCSF Chimera *(60)*, and used it to create a set of 40 Å low-pass filtered projections for reference-based 2D classifications *(56)* of the large set of particles picked with the Raptor-Rag-Ragulator complex in mind (1,293,759). Three rounds of reference-free 2D classifications were needed to arrive at 49,837 particles in 21 classes that most closely resembled a Raptor-free sub-complex. Any remaining traces of Raptor were further removed in 3D classifications, leaving only 14,186 particles that belonged to two out of eight 3D classes. The final 3D-refined map was sharpened, and estimated to extend to 8.9 Å resolution, according to the 'gold standard' FSC of 0.143. The directional resolution of the final map was measured with the 3DFSC server *(59)* (Fig. S8).

Model building and refinement – Raptor-Rag-Ragulator

Coordinates from the following structures were docked into the cryo-EM reconstruction with UCSF Chimera *(60)*: 6BCX for Raptor *(31)*, 3R7W, 3LLU for Rag GTPases *(28)*, and 5X6V for Ragulator *(25)*. The initial model was adjusted by hand and rebuilt using Coot *(61)*, and then refined with phenix.real_space_refine *(62)* to improve fitting, geometry and atom clashes. Model quality was evaluated using MolProbity *(63)* and EMRinger *(64)*. The final model contains 2211 amino acids, one molecule of GTP bound to RagA, one molecule of GDP bound to RagC, and one magnesium ion bound to RagA (Table S2). Figures of the map and the final model were rendered in UCSF Chimera *(60)* and ChimeraX *(65)*.

Chromatographic analysis of bound nucleotides

The nucleotide extraction protocol was modified from *(66)*. 3 nmol of purified Rag dimers were denatured with 0.5% (v/v) perchloric acid, and the pH was raised with 140 mM sodium acetate (pH 4). The resulting protein precipitation was clarified with a 5 min spin at 21,000×g, and the supernatant was analyzed on a Waters Ultra-High Performance Liquid Chromatography (UPLC) Dual-Pump System. Chromatographic separations were performed using a C18 column (ACQUITY UPLC BEH C18, 130Å, 1.7 µm, 2.1 mm X 50

mm, Waters #186002350) with the following reverse-phase buffer system: Buffer A [100 mM sodium phosphate, pH 6.0, 10 mM tetrabutylammonium bromide (TBAB)] and Buffer B [40:60 acetonitrile - water (v/v)]. Nucleotides were eluted at a flow-rate of 0.3 mL/min, with a 4-minute step-gradient as follows: 95% A / 5% B (min 0.0 – 1.0), 85% A / 15% B (min 1.0 – 2.0), 80% A / 20% B (min 2.0 – 2.5), 50% A / 20% B (min 2.5 – 3.0), 95% A / 5% B (min 3.0 – 4.0).

cDNA transfections

HEK-293T cells were cultured in DMEM, supplemented with 10% IFS and 2 mM glutamine, and maintained at 37°C and 5% CO_2. A seed culture of 1.5 x 10^6 cells was started 24 h before transfections. The cells were transfected with a preformed complex of PEI and 5 µg of expression plasmids in a 3:1 mass ratio. Experiments were performed 36 – 48 h after transfection.

Lentivirus production and lentiviral infections

HEK-293T cells were seeded in 6-well plates at a density of 7.5 x 10^5 cells per mL of DMEM supplemented with 20% IFS. 24 h later, XTremeGene 9 (Roche) was used to co-transfect the cells with VSV-G envelope and CMV ΔVPR packaging plasmids with either pLJC5 plasmids containing cDNAs, or with the pLentiCRISPRv2 plasmid containing indicated guide sequences. 12 h post transfection, the culture media was exchanged to fresh, and 36 h post exchange, the virus-containing supernatant was collected and clarified by passing through a 0.45 µm filter. Target cells were plated in 6-well plates with 8 µg/mL polybrene, and incubated with virus-containing media. Infections with pLentiCRISPRv2 were spinfected at 800×g for 45 minutes at 37°C. 48 h later, the culture media was exchanged to fresh, and supplemented with 0.5 µg/mL puromycin for selection.

Immunoprecipitation

Harvested cells were rinsed with cold PBS and resuspended in lysis buffer [40 mM HEPES pH 7.4, 2.5 mM $MgCl_2$, 1% Triton X-100, 10 mM β-glycerol phosphate, 10 mM pyrophosphate, protease inhibitor cocktail (Roche #11873580001, 1 tablet per 50 mL)]. To interrogate interactions between Raptor and mTOR, the 1% Triton X-100 in lysis buffer was exchanged for 0.3% CHAPS – in order to preserve the integrity of the mTOR-containing complexes. The resulting cell lysate was cleared by centrifugation (21,000×g for 10 minutes at 4°C). Cell lysate samples were prepared by supplementation with SDS

sample buffer (5X stock: 250 mM Tris, pH 7.0, 10% SDS, 25% glycerol, 0.5 M DTT, and 0.01% bromophenol blue), resolved by an 8%-16% SDS-PAGE, and then further analyzed by immunoblotting. Clarified lysates were supplemented with either 25 µL of anti-Flag affinity resin (50% slurry, Sigma #A2220) or magnetic anti-HA beads (50% slurry, Pierce #88836), and then incubated for 2 h at 4°C with shaking. Immunoprecipitates were then washed with 20 CV of lysis buffer and 40 CV of lysis buffer supplemented with 500 mM NaCl. Immunoprecipitated proteins were eluted from beads through supplementation with 50 µL of SDS sample buffer, and heat-denaturation for 5 minutes at 95°C. The resulting samples were resolved by an 8%-12% SDS-PAGE, and further analyzed by immunoblotting.

Generation of CRISPR/Cas9 genetically modified cells with loss of RagA and RagB

The following sense (S) and antisense (AS) oligonucleotides encoding the guide RNAs were cloned into the pX458 plasmid:

sgRagA_guide1_S: caccgGGAGTGTTCCACGTCAATGG

sgRagA_guide1_AS: aaacCCATTGACGTGGAACACTCCc

sgRagB_guide1_S: caccgGAGACATACACACCTTATAG

sgRagB_guide1_AS: aaacCTATAAGGTGTGTATGTCTCc

A seed culture of 5 x 10^5 cells in 6-well plates was started 24 h before transfections. XtremeGene 9 was used to transfect each well with 1 µg of the guide-containing pX458 construct and 4 µg of empty pRK5 backbone. 48 h post transfections, single cells were sorted for high GFP expression with a flow cytometer into the 96-well plates containing 200 µL of DMEM supplemented with 30% IFS.

Generation of CRISPR/Cas9 genetically modified cells with a partial loss of Raptor

The following sense (S) and antisense (AS) oligonucleotides encoding the guide RNA *(67)* were cloned into the pLentiCRISPRv2 plasmid:

sgRPTOR_guide7_S: caccgGGAGCAGCTTCAGCACGTAG

sgRPTOR_guide7_AS: aaacCTACGTGCTGAAGCTGCTCCc

Lentivirus production and infection were done as described above. 48 h post puromycin selection, single cells were sorted with a flow cytometer into 96-well plates containing 200 µL of DMEM supplemented with 30% IFS. All clones that grew into a population were tested for Raptor expression and mTORC1 activity as indicated by phosphorylation of its substrate S6K1, by immunoblotting. One of the clones showed decreased Raptor

expression and diminished mTORC1 activity. Genomic DNA was extracted from this hypomorphic Raptor cell line, and used for PCR amplification with the following oligonucleotides of the genomic region of Raptor encompassing the guide RNA site:

Seq_Primer_S: CTGAAGATAGCACCGCAGCTGG

Seq_Primer_AS: GTTCACATCACTCTGGGGAGGAG

The amplified 250 bp product was cloned into pCR-Blunt II-TOPO vector (Invitrogen) followed by transformation into DH5α competent cells. 24 bacterial colonies were selected and grown in liquid culture, harvested, and used for DNA extraction. Sanger sequencing revealed two types of mutations: (1) deletion of Y475, and (2) deletion of exon 13. No wild type Raptor sequence was detected. Exon 13 encodes a part of Raptor responsible for the recruitment of mTORC1 substrates, S6K1 and 4EBP1 *(31)*, and Y475 is a highly conserved residue in this region that has a key role in substrate recognition. These mutations, as well as the partial loss of Raptor expression, most likely explain the defect in mTORC1 activity we observe in this Raptor hypomorph cell line.

Immunofluorescence assays

Immunofluorescence assays were performed as described previously *(68)*. A seed culture of 4 x 10^5 cells was started on fibronectin-coated glass coverslips in 6-well plates. After 24 hours, the slides were rinsed once with PBS and fixed with a 4% paraformaldehyde – PBS solution for 15 minutes at 25°C. The slides were then rinsed three times with PBS and the cells permeabilized with a 0.05% Triton X-100 – PBS solution for 5 minutes at 25°C. The slides were rinsed three times with PBS and then blocked for 1 h in Odyssey blocking buffer (Li-Cor #927-40000) at 25°C. The slides were incubated with primary antibodies [mTOR (CST; 1:300), LAMP2 (SCBT; 1:300)] in Odyssey blocking buffer for 16 h at 4°C, and then rinsed three times with PBS followed by 1 h incubation with secondary antibodies produced in donkey (diluted 1:1000 in Odyssey blocking buffer) at 25°C in the dark. After three washes with PBS, the slides were mounted on glass coverslips using Vectashield (Vector Laboratories) containing DAPI. Images were acquired on a Zeiss AxioVert200M microscope with a 63X oil immersion objective and a Yokogawa CSU-22 spinning disk confocal head with a Borealis modification (Spectral Applied Research / Andor) and a Hamamatsu ORCA-ER CCD camera. The MetaMorph software package (Molecular Devices) was for image acquisition. The excitation lasers used to capture the images were 405 nm, 488 nm, 561 nm and 640 nm. The secondary antibodies used in

this study were conjugated with the following dyes: Alexa488 for mTOR staining (excitation at 488 nm), and Alexa568 for LAMP2 staining (excitation at 561 nm).

Equilibrium GTP binding assays

Assays were performed as described previously *(15)*. Newly thawed Rag GTPases produced in human cells were first centrifuged for 1 h at 200,000×g to remove any precipitated protein, and their concentration was redetermined. Increasing concentrations of Rag GTPases, ranging from 5 nM to 2 μM (on a log-scale), were mixed with a trace amount (1 nM) of α-^{32}P-GTP (Perkin-Elmer) in assay buffer (50 mM HEPES, pH 7.4, 100 mM potassium acetate, 2 mM $MgCl_2$, 2 mM DTT, 0.1% CHAPS). The reaction mixtures were incubated for 4 h at 4°C (until equilibrium was reached), and then directly spotted onto a chilled metal block covered with Parafilm. 0.3 J of UV light (254 nm) was applied to the droplets, to induce non-specific, zero-distance cross-linking between the nucleotide and the protein. The resulting protein-nucleotide cross-links were analyzed by 12% Tris-Glycine SDS-PAGE. After fixing and drying, the gel was exposed to a phosphor imaging screen, and the radioactive signal visualized on a Typhoon scanner (GE Healthcare).

Molecular dynamics simulations

In preparation for molecular dynamics simulations, the disordered parts of the switch machinery in our cryo-EM structure of RagC•GDP were modeled based on the crystal structure of RagC•GTP (PBDID: 3LLU). This model of RagC•GDP with a complete set of switches served as a starting point for simulations. All MD simulations were performed using GROMACS 2019.3 *(69)* and the CHARMM36 force field *(70)*. The GTPase domain of RagC•GDP from our Raptor-Rag-Ragulator cryo-EM structure and the GTPase domain of RagC•GTP (PBDID: 3LLU) were placed in dodecahedral simulation boxes of 257 nm^3 volume with periodic boundary conditions. The proteins were solvated by adding explicit SPC water molecules (RagC•GDP: 7,342, RagC•GTP: 7,300). Na^+ and Cl^- ions were added to the final concentration of 0.1 M. The system was first minimized with the steepest descend algorithm, equilibrated in constant temperature (NVT, 300 K) for 100 ps, and followed by equilibration in constant temperature and pressure (NPT, 1 bar) for 500 ps. The pressure was controlled with an isotropic Parrinello-Rahman barostat *(71)* applied to the entire system with a time constant of 2.0 ps and compressibility of 4.5×10^{-5} bar^{-1}. The temperature was controlled with two velocity rescaling thermostats *(72)* applied to the protein and solvent, with a time constant of 0.1 ps. Electrostatic interactions were

calculated using the particle-mesh Ewald summation method (73). Ten replicate simulations of 100 ns, and one longer simulation of 1 μs duration were calculated using the Whitehead Institute's computing facility.

Captions for Movies S1 to S5

Movie S1.

The experimental electron density (left) and the corresponding molecular model (right) of the Raptor-Rag-Ragulator complex.

Movie S2.

Variability analysis of the Raptor-Rag-Ragulator complex structure. Note the swinging motion of the two halves of the complex.

Movie S3.

Following GTP hydrolysis, strand β2 of the RagC interswitch changes its register by two residues, such that the β2-β3 interswitch loop becomes more compact. This interswitch movement has further implications on the position of the GTPase domain in the context of the Rag heterodimer (see Fig. 4C and 4D). Molecular dynamics simulations revealed that the switch machinery of RagC in the GTP-bound state is fairly stable. However, upon hydrolysis to GDP, and following the interswitch shift, a large portion of the RagC switch machinery becomes highly dynamic (switch I, interswitch strand β2 and a part of switch II). Indeed, all those dynamic switch regions are also found disordered in our cryo-EM structure of the Raptor-Rag-Ragulator complex.

Movie S4.

Raptor is unable to form a lasting interaction with Rag GTPases in the GTP-GTP nucleotide state. Following GTP hydrolysis by RagC, an extra space is made in between the two GTPase domains of the Rags. The Raptor claw can detect the availability of this space, and therefore by extension – detect the nucleotide state of RagC. Please note that the modeled GTPase domain of RagC•GTP in this movie was not corrected for its interswitch-induced shift relative to the CRD.

Movie S5.

Dimeric mTOR forms a large protein assembly through its interaction with Raptor (to form mTORC1), and through Rag-Ragulator that anchors it to the lysosomal surface. Such positioned mTORC1-Rag-Ragulator supercomplex becomes allosterically activated through its association with Rheb GTPases.

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