Mechanism of arginine sensing by CASTOR1 upstream of mTORC1

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The mechanistic Target of Rapamycin Complex 1 (mTORC1) is a major regulator of eukaryotic growth that coordinates anabolic and catabolic cellular processes with inputs such as growth factors and nutrients, including amino acids1–3. In mammals arginine is particularly important, promoting diverse physiological effects such as immune cell activation, insulin secretion, and muscle growth, largely mediated through activation of mTORC1 (refs 4–7). Arginine activates mTORC1 upstream of the Rag family of GTPases8, through either the lysosomal amino acid transporter SLC38A9 or the GATOR2-interacting Cellular Arginine Sensor for mTORC1 (CASTOR1)9–12. However, the mechanism by which the mTORC1 pathway detects and transmits this arginine signal has been elusive. Here, we present the 1.8 Å crystal structure of arginine-bound CASTOR1. Homodimeric CASTOR1 binds arginine at the interface of two Aspartate kinase, Chorismate mutase, TyrA (ACT) domains, enabling allosteric control of the adjacent GATOR2-binding site to trigger dissociation from GATOR2 and downstream activation of mTORC1. Our data reveal that CASTOR1 shares substantial structural homology with the lysine-binding regulatory domain of prokaryotic aspartate kinases, suggesting that the mTORC1 pathway exploited an ancient, amino-acid-dependent allosteric mechanism to acquire arginine sensitivity. Together, these results establish a structural basis for arginine sensing by the mTORC1 pathway and provide insights into the evolution of a mammalian nutrient sensor.

To understand the molecular mechanisms through which CASTOR1 detects the presence of arginine and signals it to mTORC1, we determined the crystal structure of arginine-bound CASTOR1 to 1.8 Å resolution (Extended Data Table 1). Our findings show that CASTOR1 forms a rod-shaped homodimer, with the monomers associated in a side-by-side manner and rotated 180° with respect to each other (Fig. 1a). Although sequence analysis of CASTOR1 predicted the presence of two ACT domains12,13, the structure reveals that each monomer actually contains four tandem ACT domains. ACT1 displays the canonical βαββαββαβACT domain topology14,15, whereas ACT2 contains two additional β-strands and ACT3 and ACT4 each lack the final β-strand. (Fig. 1a and Extended Data Fig. 1a).

The dimerization interface buries around 950 Å² of surface area at the intersection between the α1 helix of ACT1 and the α5 helix of ACT3 (Fig. 1b). Two inward-facing isoleucine residues of each monomer (Ile28 and Ile202) form the hydrophobic core of the symmetrical interface, flanked on each side by tyrosine–histidine pairs (His25 and Tyr207) that form both π-stacking and hydrogen-bond contacts with the opposing monomer (Fig. 1b). To understand the importance of dimerization in CASTOR1 function, we generated constitutively monomeric mutants of CASTOR1 (Y207S and I202E; Fig. 1c). Notably, although dimerization is dispensable for arginine binding (Extended Data Fig. 2a), these mutants interacted weakly with CASTOR1 and led to robust inhibition of mTORC1 signalling in cells (Fig. 1c and Extended Data Fig. 2b). This finding indicates that CASTOR1 must be dimeric to robustly inhibit GATOR2 upon arginine starvation.

CASTOR1 binds arginine through a narrow pocket at the interface of ACT2 and ACT4, distal to the dimerization interface (Fig. 1a, 2a, b). The side chain of arginine projects towards the β15 loop, a loop connecting β15 and β16, where the backbone carbonyls of Thr300, Phe301, and Phe303 coordinate the guanidinium group of arginine (Fig. 2a). Immediately adjacent to the β15 loop, the anionic side chain of Asp304 forms an additional stabilizing salt bridge with the cationic arginine side chain (Fig. 2a). On the opposite side of the pocket, the hydroxyl side chain of Ser111 and the backbone carbonyl of Val112 in the α3 loop anchor the free amino group of arginine in place, while the free carboxyl group points towards a water-filled cavity that separates it from ACT2 (Fig. 2a, b). Mutation of either Ser111 or Asp304 (S111A, D304A) abolished the arginine-binding ability of CASTOR1 in vitro, highlighting the critical role of these contacts in arginine sensing by CASTOR1 (Fig. 2c). Furthermore, when expressed in HEK-293T cells, these mutants bound constitutively to GATOR2 and strongly inhibited mTORC1 signalling even in the presence of arginine (Fig. 2d).

Together, these data explain the molecular determinants of specificity in the CASTOR1–arginine interaction. While Ser111 fixes the position of the free amine, the location of the β15 loop and Asp304 sets a strict length requirement for the bound ligand (Extended Data Fig. 3a). In addition, the positions of the three hydrogen-bond-donating nitrogen atoms in the guanidinium group facilitate contacts with both the carbonyl oxygen atoms in the β15 loop and the side chain of Asp304 (Fig. 2a). Finally, the gap behind the free carboxyl group of arginine suggests that CASTOR1 can tolerate ligands with modifications to that functional group (Fig. 2b). We tested these predictions by investigating the ability of various arginine analogues to disrupt the CASTOR1–GATOR2 interaction in vitro (Fig. 2e and Extended Data Fig. 3b). Consistent with our structural analysis, while the carboxy-modified arginine–methyl ester triggered full dissociation of CASTOR1 from GATOR2, compounds with alterations to the guanidinium group, α-amine, or the length of the side chain had no effect.

In addition to the main pocket contacts described above, a highly conserved, glycine-rich loop connecting β14 and α7 in ACT4 (β14 loop, residues 269–280) wraps over the arginine pocket, fully burying the bound ligand (Figs 2a, 3a and Extended Data Fig. 1a). The β14 loop forms several hydrogen bonds with arginine through the backbone amides of Gly279 and Ile280, as well as the backbone oxygen atoms of Gly274 and Glu277 (Figs 2a, 3a). The ordered conformation of the β14 loop also places it just along the ACT2–ACT4 interface, enabling it to form several intramolecular contacts with residues in ACT2 (Fig. 3a). Cys278 forms hydrogen bonds with the backbones of Val110 and S111 in the α3 loop, while Asp276 forms a salt bridge with Arg126. In addition, Glu277 extends in the opposite direction to form another salt bridge with His175 (Fig. 3a). Thus, the β14 loop facilitates
the formation of numerous inter-ACT-domain contacts in the presence of arginine. Indeed, the arginine and β14 loop contribute about 40% of the total buried surface area in the ACT2–ACT4 interface of the arginine-bound structure (390 Å² out of 980 Å²).

The glycine-rich β14 loop is predicted to have a high propensity for disorder. Our structure suggests that these inter-ACT-domain contacts could stabilize it in an ordered conformation over the bound arginine.

Indeed, mutation of key residues in both the β14 loop (D276A, E277A, C278A) and the adjacent ACT domains (R126A, H175A) significantly reduced the arginine-binding capacity of CASTOR1 (Fig. 3b, c), indicating that the inter-ACT-domain contacts formed by the β14 loop are required for arginine sensing by CASTOR1. In addition, we found that the N-terminal (ACT1 and ACT2) and C-terminal (ACT3 and ACT4) halves of CASTOR1 associated in both an arginine- and

Figure 1 | Architecture of human CASTOR1. a, Two orthogonal views of the CASTOR1 homodimer (ribbon diagram), with ACT-domains 1–4 coloured in green, purple, wheat, and pink, respectively. The bound arginine is shown in yellow. Disordered regions not observed in the crystal structure are omitted. b, View of the CASTOR1 dimerization interface, with side chains of key residues represented in stick form. c, Dimerization-deficient CASTOR1 Y207S and I202E mutants display weaker interactions with endogenous GATOR2. HEK-293T cells transiently expressing FLAG-tagged CASTOR1 wild type (WT) and the indicated haemagglutinin (HA)-tagged constructs were starved of arginine for 50 min and, where indicated, re-stimulated for 10 min. HA-immunoprecipitates were generated from cell lysates and analysed by immunoblotting for the indicated proteins. Mis was used as a representative GATOR2 component.
The CASTOR1 D276A, R126A, E277A, H175A, and C278A mutants constitutively bind GATOR2 in cells. HEK-293T cells transiently expressing the indicated HA-tagged constructs were starved of arginine for 50 min and, where indicated, re-stimulated for 10 min. HA-immunoprecipitates were prepared and analysed as in Fig. 1c. The CASTOR1 ACT1-2 (residues 1–169) and CASTOR1 ACT3–4 (169–329) associate in an arginine- and 314-loop-dependent manner. The CASTOR1 D276A, R126A, E277A, H175A, and C278A mutants constitutively bind GATOR2 in cells. HEK-293T cells transiently expressing the indicated HA-tagged constructs were starved of arginine for 50 min and, where indicated, re-stimulated for 10 min. HA-immunoprecipitates were prepared and analysed as in Fig. 1c. The CASTOR1 ACT1-2 (residues 1–169) and CASTOR1 ACT3–4 (169–329) associate in an arginine- and 314-loop-dependent manner. In addition to CASTOR1, human cells express a related protein, CASTOR2, which shares 63% sequence identity with CASTOR1 but does not bind arginine12. Although the regions of CASTOR1 that are directly involved in arginine binding are well conserved (Extended Data Fig. 1a), we identified residues along the ACT2–ACT4 interface (His108 to Val110) that differ between CASTOR1 and CASTOR2 (Extended Data Fig. 1a, b). Replacing these residues in CASTOR1 with those from CASTOR2 abrogated arginine binding in vitro and converted CASTOR1 to a nearly-constitutive GATOR2-interactor in cells, resembling CASTOR2 (Extended Data Fig. 4b–d). Notably, these residues immediately precede Ser111 and form a hydrogen bond with Cys278 in the 314 loop (Fig. 3a and Extended Data Fig. 4a), suggesting that their identity may be critical for the proper positioning of the α3 loop to enable arginine binding and/or the association of ACT2 and ACT4. The corresponding mutation in CASTOR2 (QNI108–110HHV), however, was not sufficient to confer arginine-binding ability, suggesting that additional amino acid differences also contribute to this functional difference (Extended Data Fig. 4d).

To understand how arginine induces dissociation of CASTOR1 from GATOR2, we identified five highly conserved sites in CASTOR1 that are required for its interaction with GATOR2 (Y118, Q119, D121, E261 and D292; Fig. 4a and Extended Data Fig. 1a). Importantly, these mutants still bind arginine in vitro and homodimerize when expressed in cells (Extended Data Fig. 5a, b). Notably, these residues cluster along the surface of the ACT2–ACT4 interface, adjacent to the arginine-binding pocket but on the opposite face of the protein (Fig. 4b, c). Glu261 and Asp292 are closely linked to the 314 loop, separated only by α14 and α7, respectively (Fig. 4c). Furthermore, the critically important residue Asp121 is buried in the ACT2–ACT4 interface, potentially explaining why the arginine-bound conformation of CASTOR1 does not interact with GATOR2 (Fig. 4c).

Together, these results suggest a model in which arginine binding arranges the glycine-rich 314 loop in a conformation that enables the intramolecular association of ACT2 and ACT4 (Fig. 3a–d). The association of these domains would alter the position and exposure of the residues required for GATOR2 binding, which also lie along the ACT2–ACT4 interface (Fig. 4a–c), thereby triggering the dissociation of CASTOR1 from GATOR2 and the subsequent activation of mTORC1 (Fig. 4e).

The observation that CASTOR1 inhibits mTORC1 signalling and interacts with GATOR2 in an arginine-sensitive manner suggests that CASTOR1 may regulate mTORC1 by inhibiting GATOR2, a mechanism analogous to that of the recently identified leucine sensor Sestrin2 (refs 16–19). Using our GATOR2-binding-deficient mutants, we were able to test this hypothesis directly. In contrast to wild-type CASTOR1, the GATOR2-binding-deficient YQ118–119AA and D121A mutants both failed to inhibit mTORC1 signalling in cells (Fig. 4d). Moreover, owing to their ability to dimerize with endogenous CASTOR1, these mutants also functioned as dominant negatives, rendering mTORC1 fully resistant to arginine starvation (Fig. 4d). Thus, the CASTOR1–GATOR2 interaction is required to signal arginine deprivation to mTORC1.

Although defined by their common topology, ACT domains are highly diverse in sequence and form a wide range of structural assemblies14,15. Comparison of our structure with other ACT-domain-containing proteins in the Protein Data Bank (PDB) revealed that CASTOR1 shares substantial structural homology with the allosteric regulatory domains of bacterial aspartate kinases, including those found in *Escherichia coli* (AKeco) and cyanobacteria (AKsyn)20,21 (Fig. 5a and Extended Data Fig. 6a). Aspartate kinases catalyse the first step of a metabolic pathway that synthesizes several amino acids, including lysine, and display allosteric inhibition when downstream products bind to their regulatory domains22. Notably, AKeco binds lysine through pockets that bear a striking resemblance to the arginine-binding pocket of CASTOR120 (Fig. 5b). Furthermore, AKeco residues Arg305, Glu346, and Val347, which correspond to the positions of the critical GATOR2-binding residues Glu261, Tyr118, and Glu119, respectively, participate directly in the lysine-dependent inhibition of the kinase domain in AKeco20 (Extended Data Fig. 6b). Thus, the overall structure, mode of amino-acid binding and likely allosteric
Figure 4 | The GATOR2 binding site of CASTOR1 is at the ACT2–ACT4 interface and is required for signalling arginine deprivation to mTORC1. a, The CASTOR1 D292A, E261A, D121A, and YQ118–119AA mutants are deficient in GATOR2 binding. HA-immunoprecipitates prepared from arginine-starved HEK293T-cells transiently expressing the indicated HA-tagged constructs were analysed as in Fig. 1c. b, Solvent-exposed surface view of the CASTOR1 homodimer highlighting the GATOR2-binding sites (red). Residue E261 is in a partially disordered loop and not visible in one monomer (left). c, Cross-sectional view of the ACT2–ACT4 interface showing the positions of the critical GATOR2-binding residues relative to the bound arginine (yellow) and the β14 loop. d, The GATOR2-binding-deficient YQ118–119AA and D121A mutants of CASTOR1 fail to inhibit the mTORC1 pathway and render cells insensitive to arginine starvation. HEK–293T cells were transiently transfected with FLAG–S6K1 and the indicated HA-tagged constructs. FLAG-immunoprecipitates were prepared and analysed as in Fig. 1d. e, A model of how arginine releases CASTOR1 from GATOR2 to activate mTORC1.

Figure 5 | Insights into the evolution of arginine sensing by CASTOR1. a, Top, a ribbon view of human CASTOR1 dimer (pink and purple) and AKeCo dimer (blue and yellow; PDB ID 2J0X). Bottom, a ribbon view of the human CASTOR1 monomer (left) and the regulatory domain from AKeCo (right). b, Comparison of the arginine-binding pocket in human CASTOR1 with the lysine-binding pocket in AKeCo. Arginine and lysine are shown in yellow and orange, respectively. Hydrogen bonds and salt bridges are shown as black dashed lines. c, Phylogenetic distribution of aspartate kinase (orange) and CASTOR1 homologues (purple). d, Model of the evolution of CASTOR1 from the regulatory domain of an ancestral aspartate kinase.
analouges suggest that our structure may be useful for predicting compounds that can modulate arginine sensing by CASTOR1 in vivo. As the deregulation of mTORC1 is common in a number of human diseases, including cancer26,27, the identification of novel pharmacological regulators of mTORC1 activity is of particular interest.

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

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Materials. Reagents were obtained from the following sources: HRP-labelled anti-rabbit secondary antibody from Santa Cruz Biotechnology; antibodies to phospho-T389 S6K1, S6K1, Mios and the FLAG epitope from Cell Signalling Technology; antibodies to the haemagglutinin epitope from Bethyl laboratories; antibody to raptor from Millipore. All antibodies used have been published previously. FLG–M2 affinity gel and amino acids from Sigma Aldrich; RPMI without leucine, arginine, or lysine from Pierce; DMEM from SAFC Biosciences; XhoI, EcoRI9 and Complete Protease Cocktail from Roche; inactivated fetal calf serum (IFS) from Invitrogen; [3H]-labelled arginine from American Radiolabelled Chemicals.

Protein production and purification. Full-length, codon-optimized human CASTOR1 was N-terminally fused with a human rhinovirus 3C protease–cleavable His10–Arg8–ScSUMO tag and cloned into a pET-Duet-1 bacterial expression vector. This vector was transformed into E. coli LOBSTR (DE3) cells (Kerafast). Cells were grown at 37 °C to 0.6 optical density (OD), then protein production was induced with 0.2 mM IPTG at 18 °C for 12–14 h. Cells were collected by centrifugation at 6,000g, re-suspended in lysis buffer (50 mM potassium phosphate, pH 8.0, 500 mM NaCl, 30 mM imidazole, 3 mM 3-mercaptoethanol (UME) and 1 mM PMSF) and lysed with a cell disruptor (Constant Systems). The lysate was cleared by centrifugation at 10,000g for 20 min. The soluble fraction was incubated with Ni-Sepharose 6 Fast Flow beads (GE Healthcare) for 30 min on ice. After washing of the beads with lysis buffer, the protein was eluted in 250 mM imidazole, pH 8.0, 150 mM NaCl and 3 mM UME. The Ni eluate was diluted 1:1 with 10 mM potassium phosphate, pH 8.0, 0.1 mM EDTA and 1 mM dithiothreitol (DTT), and was subjected to cation-exchange chromatography on a 5 ml SP sepharose fast flow column (GE Healthcare) with a linear NaCl gradient. The eluted CASTOR1 was then incubated with 3C protease and dialysed overnight at 4 °C into 10 mM potassium phosphate, pH 8.0, 150 mM NaCl, 0.1 mM EDTA and 1 mM DTT, followed by a second cation-exchange chromatography run on an SP sepharose fast flow column (GE Healthcare) with a linear NaCl gradient. The protein was further purified via size-exclusion chromatography on a Superdex S200 16/60 column (GE Healthcare) equilibrated in running buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1 mM EDTA and 1 mM DTT). Selenomethionine (SeMet)–derivatized CASTOR1 was prepared as described previously and purified as the native version, except that the reducing-agent concentration (DTT) was 5 mM in all buffers.

Cristalization. Purified CASTOR1 was concentrated to 6 mg/ml and incubated in 2 mM arginine for >1 h before setting crystal trays. Crystals were grown at 18 °C by hanging-drop vapour diffusion with 1 μl of protein at 6 mg/ml mixed with an equal volume of reservoir solution containing 0.1 M sodium acetate pH 5.0, 0.25 M ammonium acetate, and 22.5% PEG 3350. Selenomethionine–derivatized CASTOR1 was crystallized in 0.1 M Bis-Tris pH 5.6, 0.25 M ammonium acetate, and 22.5% PEG3350. Crystals were cryoprotected in mother liquid supplemented with 20% (v/v) ethylene glycol.

Data collection and structure determination. Data collection was performed at the Advanced Photon Source end station 24-IDC at Argonne National Laboratory, at 100 K. All data-processing steps were carried out with programs provided through SRgird. Data reduction was performed with HKL2000. A complete native data set was collected to 1.8 Å (at wavelength 0.9792 Å) and a complete SeMet data set, at the selenium peak wavelength (0.9792 Å), was collected to 2.2 Å. The phase problem was solved using single-wavelength anomalous dispersion (SAD) and selenium positions were determined in HySS, run as part of the PHENIX AutoSol program22, for the SeMet data set (space group P212121, 4 molecules per asymmetric unit). An interpretable 2.2 Å experimental electron density map was obtained, and manual model building was carried out in Coot35. Subsequent refinement was carried out with the superior 1.8 Å native data set using phenix. refein to refine at 17.2% 20.4%. Ramachandran statistics in the final model are 99% favoured, 1% allowed, and 0% outlier.

Structural analysis. Protein–protein and protein–ligand interfaces were analysed using PDBesPPA. NCBI’s Vector Alignment Search Tool (VAST)35 was used to identify structurally related proteins in the PDB. The multiple sequence alignment (MSA) was generated in Jalview36 with the T-Coffee alignment algorithm37. NCBI’s Vector Alignment Search Tool (VAST)35 was used to identify structurally related proteins in the PDB. The multiple sequence alignment (MSA) was generated in Jalview36 with the T-Coffee alignment algorithm37.

Following immunoprecipitation, the beads were washed four times with lysis buffer containing 500 mM NaCl. Immunoprecipitated proteins were denatured by the addition of 50 μl of sample buffer and boiling for 5 min as described46, resolved by 8–16% SDS–PAGE, and analysed by immunoblotting.

For co-transfection experiments in HEK-293T cells, 2.5 million cells were plated in 10 cm culture dishes. Twenty-four hours later, cells were transfected using the polyethylenimine method with the pRK5-based CD expression plasmids indicated in the following amounts: 50 ng CASTOR1–HA (wild-type or mutant), 50 ng CASTOR1–FLAG, 1 μg HA-tagged, or 2 μg S6K. For in vitro dissociation experiments, 50 ng of wild-type CASTOR1–HA was transfected into HEK-293T cells. The total amount of plasmid DNA in each transfection was normalized to 5 μg with empty pRK5. 36–48 h after transfection, cells were lysed as described above.

For experiments that required amino acid starvation or re-stimulation, cells were treated as previously described. Briefly, cells were incubated in arginine-free RPMI for 50 min and then re-stimulated with 500 μM arginine for 10 min.

Arginine binding assay. Five million HEK-293T cells were plated on a 15 cm plate four days before the experiment. Twenty-four hours after plating, the cells were transfected via the polyethylenimine method with the pRK5-based CD expression plasmids indicated in the figures in the following amounts: 15 μg FLAG–Rap2A, 500 ng FLAG–CASTOR1 (wild-type or mutant). The total amount of plasmid DNA in each transfection was normalized to 15 μg total DNA with empty pRK5. Forty-eight hours after transfection cells were lysed as previously described. If multiple samples of the same type were represented in the experiment, the cell lysates were combined, mixed, and evenly distributed amongst the relevant tubes.

Anti-FLAG beads were blocked by rotating in 1 mg/ml bovine serum albumin (BSA) for 20 min at 4 °C, then washed twice in lysis buffer and re-suspended in an equal volume of lysis buffer. 30 μl of bead slurry was added to each of the clarified cell lysates, and the samples were incubated as previously described. After immunoprecipitation, the beads were washed as previously described and incubated for one hour on ice in cytosolic buffer (0.1% Triton, 40 mM HEPES pH 7.4, 10 mM NaCl, 150 mM KCl, 2.5 mM MgCl) with the appropriate amount of [3H]-labelled arginine and cold arginine. At the end of one hour, the beads were aspirated dry and rapidly washed three times with cytosolic buffer. The beads were aspirated dry again and resuspended in 85 μl of cytosolic buffer. Each sample was mixed well and three 10 μl aliquots were quantified separately using a TriCarb scintillation counter (PerkinElmer). This process was repeated in pairs for each sample, to ensure similar incubation and wash times for all samples analysed across different experiments.

In vitro CASTOR1–GATOR2 dissociation assay with arginine analogues. HEK-293T were transfected with HA–CASTOR1 constructs as described above. 48 h after transfection, cells were starved for all amino acids for 50 min, lysed and subjected to anti-FLAG immunoprecipitation as described previously. The CASTOR1–GATOR2 complexes immobilized on the haemagglutinin beads were washed twice in lysis buffer with 500 mM NaCl, then incubated for 20 min in 1 μl of cytosolic buffer with 400 μM of the indicated compound. The amount of GATOR2 and CASTOR1 that remained bound was assayed by SDS–PAGE and immunoblotting as described previously.

Cell lines and tissue culture. HEK-293T cells were maintained at 37 °C and 5% CO2 and cultured in DMEM 10% IFS supplemented with 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). HEK-293T cells were obtained from the American Type Culture Collection (ATCC) and were free of mycoplasma contamination.

Statistical analysis. For the arginine-binding assays, two-tailed t-tests were used for comparison between two groups. All comparisons were two-sided, and P values of less than 0.005 were considered statistically significant. The data meet the assumptions of the test and the variance is similar between groups that are being statistically compared. No statistical methods were used to determine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

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Extended Data Figure 1 | Multiple sequence alignment of CASTOR1 homologues. a. Expanded Multiple Sequence Alignment of CASTOR1 homologues from various organisms. Positions are coloured white to blue according to increasing sequence identity. Secondary structure features are labelled and coloured by ACT domain as in Fig. 1a.
Extended Data Figure 2 | Dimerization-deficient CASTOR1 mutants bind arginine but fail to inhibit mTORC1 in cells. a, The dimerization-deficient CASTOR1 Y207S and I202E mutants bind arginine in vitro. FLAG-immunoprecipitates prepared from HEK-293T cells transiently expressing indicated FLAG-tagged proteins were used in binding assays with [3H]arginine as described in the Methods. Unlabelled arginine was included as a competitor where indicated. Values are mean ± s.d. for three technical replicates from one representative experiment. b, Dimerization-deficient CASTOR1 Y207S and I202E mutants fail to inhibit mTORC1. HEK-293T cells transiently expressing FLAG–S6K1 and HA-tagged wild-type, Y207S, or I202E CASTOR1 were starved of arginine for 50 min and, where indicated, re-stimulated for 10 min. FLAG-immunoprecipitates were prepared from lysates and analysed as in Fig. 1c. Phospho-S6K1 was used as an indicator of mTORC1 activity.
Extended Data Figure 3 | Model of lysine-binding in CASTOR1.

a, Comparison of the arginine-bound pocket of human CASTOR1 with a model of the pocket with lysine in place of arginine. Arginine and lysine stick representations are shown in yellow and orange, respectively. The distances in the lysine-bound model, 3.8 Å and 5.0 Å, are beyond the range of standard hydrogen bonds and salt bridges, respectively. ACT domains are labelled as in Fig. 1a. b, Chemical structures of arginine analogues used in Fig. 2e. Differences relative to L-arginine are highlighted in oranges boxes.
Extended Data Figure 4 | Differences in the arginine-binding capacities of CASTOR1 and CASTOR2. a, Multiple sequence alignment of human CASTOR1 and CASTOR2, highlighting differences in amino acid sequence that are in close proximity to arginine-binding residues in CASTOR1. b, The CASTOR1 HHV108–110QNI mutant constitutively binds GATOR2 in cells. HEK-293T cells transiently expressing HA–metap2 or the indicated HA-tagged CASTOR1 constructs were starved of arginine for 50 min and, where indicated, re-stimulated for 10 min. HA-immunoprecipitates were prepared and analysed as in Fig. 1c. c, The CASTOR1 HHV108–110QNI mutant displays reduced arginine-binding capacity in vitro. Binding assays were performed with the indicated CASTOR1 or CASTOR2 constructs and immunoprecipitates analysed as in Fig. 2c. Values are mean ± s.d. for three technical replicates from one representative experiment. d, Comparison of the CASTOR1 HHV108–110QNI mutant and wild-type CASTOR2. HEK-293T cells transiently expressing HA–metap2 or the indicated HA-tagged CASTOR1 or CASTOR2 constructs were starved of arginine for 50 min and, where indicated, re-stimulated for 10 min. HA-immunoprecipitates were prepared and analysed as in Fig. 1c.
Extended Data Figure 5 | GATOR2-binding-deficient CASTOR1 mutants still bind arginine and homodimerize. a, The CASTOR1 YQ118–119AA, D121A, E261A and D292A mutants bind arginine in vitro. FLAG-immunoprecipitates prepared from HEK-293T cells transiently expressing indicated FLAG-tagged proteins were used in binding assays with [3H]arginine as described in the Methods. Unlabelled arginine was included as a competitor where indicated. Values are mean ± s.d. for three technical replicates from one representative experiment. b, The CASTOR1 YQ118–119AA, D121A, E261A and D292A mutants dimerize in cells. HA-immunoprecipitates prepared from HEK293T-cells transiently expressing CASTOR1–FLAG and HA–metap2 or the indicated HA-tagged CASTOR1 constructs were analysed as in Fig. 1c.
Extended Data Figure 6 | Similarities between human CASTOR1 and prokaryotic aspartate kinases. a, Ribbon diagram views of human CASTOR1, AKeco (PDB ID: 2J0x) and AKsyn (PDB ID: 3L76), highlighting the different modes of dimerization. Aspartate kinases can dimerize through an interlocked-ACT domain conformation (as in AKeco) or through their kinase domains (AKsyn), both of which are distinct from the side-by-side ACT-domain dimerization in CASTOR1. b, View of AKeco depicting positions of residues R305, E346, and V347, which correspond to the positions of the GATOR2-interacting residues of CASTOR1.
## Extended Data Table 1 | Data collection and refinement statistics (SAD)

|                      | CASTOR1 + Arg Native | CASTOR1 + Arg SeMet |
|----------------------|----------------------|---------------------|
| **Organism**         | *H. sapiens*         | *H. sapiens*        |
| **PDB ID**           | 5I2C                 |                     |

### Data collection

|                      | CASTOR1 + Arg Native | CASTOR1 + Arg SeMet |
|----------------------|----------------------|---------------------|
| **Space group**      | P2₁                  | P2₁                 |
| **Cell dimensions**  |                      |                     |
| \( a, b, c \) (Å)    | 91.39, 82.60, 96.67  | 91.76, 82.35, 96.71 |
| \( \alpha, \beta, \gamma \) (°) | 90, 116.23, 90 | 90, 116.04, 90 |
| **Wavelength (Å)**   | 0.9792               | 0.9792              |
| **Resolution (Å)**   | 86.7 – 1.80          | 86.89 – 2.20        |
| **\( R_{sym} \) (%)** | 7.2 (62.8)          | 10.4 (>100)         |
| **\( I / \sigma I \)** | 25.9 (1.2)         | 22.6 (1.4)          |
| **Completeness (%)** | 97.85 (87.1)        | 98.2 (98.1)         |
| **Redundancy**       | 3 (2.5)              | 6.4 (5.9)           |
| **Anomalous Completeness (%)** | 3 (2.5)   | 96.8                |

### Refinement

|                      | CASTOR1 + Arg Native | CASTOR1 + Arg SeMet |
|----------------------|----------------------|---------------------|
| **Resolution (Å)**   | 86.71 – 1.80         |                     |
| **No. reflections**  | 116,883              |                     |
| **\( R_{work} / R_{free} \)** | 17.2%/20.4% |                     |
| **No. atoms**        | 9,872                |                     |
| Protein              | 9,012                |                     |
| Arg                  | 48                   |                     |
| Water                | 796                  |                     |
| **Average B-factors (Å)** | 40.2               |                     |
| Protein              | 40.0                 |                     |
| Arg                  | 26.8                 |                     |
| Water                | 46.4                 |                     |
| **R.m.s. deviations**|                      |                     |
| Bond lengths (Å)     | 0.007                |                     |
| Bond angles (°)      | 0.85                 |                     |

*Values in parentheses are for highest-resolution shell.*