PNAS

Supporting Information for

Conformation-specific synthetic intrabodies modulate mTOR signaling with subcellular spatial resolution

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Materials and methods

Generation of DNA constructs. All polymerase chain reactions (PCR) and ligations described here were performed using the In-Fusion HD Cloning Kit (Takara Bio). gBlock gene fragments (IDT) encoding FKBP12 and the FRB domain of mTOR (*H. sapiens* and residues corresponding to 2021-2113 for *D. melanogaster, C. elegans,* and *S. cerevisiae* TOR1) were cloned into the SmaI site of the pEKD40 expression vector bearing C-terminal 6xHis-tags as previously described (1). *H. sapiens* $mTOR^{FRB}$ was also cloned into BamHI/XhoI sites of the pHFT2 expression vector with an N-terminal 10xHis-tag and TEV cleavage site. Synthetic antibody fragment (Fab) PCR inserts were generated by PCR from unique phage clones and ligated into SalI/HindIII sites of the pRH2.2 expression vector (non-tagged) or into SphI sites of the pSFV4 expression vector (AviTag). Mutagenesis was performed according to the QuikChange Site-Directed Mutagenesis Kit (Agilent). Single chain variable fragments were generated by cloning gBlock fragments encoding sAB-R3E9 or sAB-R3H8 VL and VH domains as previously described (2) into XbaI/BamHI sites of the pSCSTa expression vector. Unique VH domains were cloned into XhoI/BamHI sites and CDR-L3 was mutated by a combination of PCR and In-Fusion ligation. C-terminal FLAG tag, eGFP, or FLAG-T2A-eGFP tags were generated by PCR. Intrabody localization tags (NES [LPPLERLTL], c-myc NLS [PAAKRVKLD], SV40 NLS [PKKKRKV], N-terminal Lyn kinase motif [GCIKSKRKDKD], and Yxx**ϕ** [YATFYSGMYQRL]) were generated by a combination of PCR and In-Fusion ligation in each of the eGFP-tagged intrabody expression vectors. Lastly, a gBlock gene fragment encoding histone 2A (H2A), mCherry, and p85 S6K1 was cloned into XbaI/BamHI sites of the pSCSTa expression vector.

Enzyme-Linked Immunosorbent Assays (ELISA). For phage ELISA, SNAP-$mTOR^{FRB}$ (50 nM) was directly immobilized for 30 minutes onto high binding 96-well microplates (Greiner Bio) followed by extensive blocking with 2% BSA for 1 hour. After 15 minutes of incubation with phage, wells were washed three times with 0.5% BSA/PBST and incubated with Protein L-HRP (Thermo Scientific) for 20 minutes. The plates were again washed and developed with TMB substrate (Thermo Scientific) followed by quenching with 10% H_3PO_4 and absorbance measurement at 450 nm. For Fab-format ELISA, targets (200 nM) were directly immobilized onto high binding 96-well microplates (Greiner Bio). The wash buffer for all Fab-format experiments was PBS supplemented with 0.05% Tween 20. Plates were washed and blocked with 1% BSA in PBS. For competitive ELISA, competitors alone were incubated first for 15 minutes. Dilutions of Fabs alone or with competitors were prepared in PBS/0.05% Tween 20/0.5% BSA and were added for 20 minutes before washing three times. Secondary detection was carried out by Protein L-HRP (Thermo Scientific) for 20 minutes. Plates were washed three times before adding TMB substrate (Thermo Scientific), quenching with 10% H_3PO_4, and measuring absorbance at 450 nm.

Surface plasmon resonance. The MASS-1 (Bruker) instrument was used for all surface plasmon resonance (SPR) analyses. Ni-NTA sensor surface was used to immobilize $mTOR^{FRB}$ via a 6xHis-tag. Fabs in two-fold dilutions were run as analytes at 30 μl/min flow rate (20°C). Raw data were corrected by double referencing. Analysis was performed with Sierra Analyser (Bruker) using a Langmuir 1:1 binding model for curve fitting. Results were then plotted using GraphPad Prism.

In vitro pulldown assay. SNAP-FKBP12 was site-specifically labeled with SNAP-Surface Alexa Fluor 488 (NEB) according to the manufacturer's protocol. The dilution and wash buffer for all pulldowns was PBS supplemented with 0.05% Tween 20 and 0.5% BSA. All incubations were performed in the dark at room temperature. Briefly, 10 nM Bio-SNAP-$mTOR^{FRB}$, 10 nM A488-SNAP-FKBP12, and 10 nM rapamycin were incubated together briefly to allow ternary complex

formation. Varying concentrations of sAB-R3E9 or sAB-R3H8 were spiked into each sample and incubated 30 minutes. Next, 10 μL of Streptavidin MagneSphere Paramagnetic Particles (Promega) were added to each samples and incubated with constant rotation for 15 mins. Samples were washed three times in 0.3 mL wash buffer supplemented with 10 nM rapamycin before elution by heating 5 mins in 20 μL 1x sample buffer for SDS-PAGE analysis. A488-SNAP-FKBP12 levels were quantified using Image Lab software to determine IC_{50} values for sAB-R3E9 and sAB-R3H8.

Immunoprecipitation and western blot analysis. For experiments with cells transfected with H2A-mChe-S6K1, the lysis buffer used was 1x RIPA (EMD Millipore) supplemented with 0.1% sodium dodecyl sulfate and 1x Halt Protease Inhibitor Cocktail (Thermo Scientific). For all other experiments, to maintain the integrity of mTORC1 assemblies, the lysis buffer used was 0.3% CHAP, 25 mM TRIS, pH 8.0, 150 mM NaCl supplemented with 5 mM EDTA and 1x Halt Protease Inhibitor Cocktail. Briefly, cells were washed one time with ice cold PBS. HeLa cells were collected by gentle scraping in ice cold PBS followed by centrifugation. Cell lysis was carried out on ice for 20 minutes with constant agitation before centrifugation at 14,000 rpm to clarify lysates. Supernatants were transferred to fresh tubes and total protein was quantified by BCA assay (Thermo Scientific). Biotinylated sAB immunoprecipitations were performed for 3 hours with rotation at 4°C. Dynabeads M-270 Streptavidin beads (Invitrogen) were equilibrated in lysis buffer before adding to lysates for 1 hour with rotation at 4°C. Beads were washed five times in lysis buffer before elution by boiling 5 minutes in SDS sample buffer containing 10 mM DTT. Anti-FLAG immunoprecipitations were performed by adding 30 μL of Anti-DYKDDDDK G1 Affinity Resin (Genscript) to lysates for 3 hours with rotation at 4°C. Samples were washed five times in lysis buffer before elution by boiling 5 minutes in SDS sample buffer. Samples were adjusted to 10 mM DTT after transferring supernatant to fresh tubes. Pan-mTOR immunoprecipitations were performed by adding anti-mTOR Rabbit IgG (Cell Signaling #2972, 1:200 dilution) to lysates and incubating overnight with rotation at 4°C. The next day, Protein A Magnetic Beads (Pierce) were equilibrated in lysis buffer and added for 1 hour with rotation at 4°C. Beads were washed five times in lysis buffer before elution by boiling 5 minutes in SDS sample buffer containing 10 mM DTT. Samples were separated via SDS-PAGE at 100V, transferred to Immobilin-P PVDF Membranes (0.45 μm, EMD Millipore), and blocked for 1 hour at room temperature in blocking buffer (5% BSA in PBS supplemented with 0.1% Tween 20). The following primary antibodies were obtained from Cell Signaling Technology: mTOR (#2972S), Raptor (#2280S), mLST8 (#3274S), Rictor (#2114S), p-S6K1^{T389} (#9234S), S6K1 (#2708T), p-Akt^{S473} (#9271T), Akt (#4691T), Actin (#4970T), Tubulin (#2148S), FLAG (#14793S), eGFP (#2956T), 4E-BP1 (#9644), p-4E-BP1$^{T37/46}$ (#2855), and p-4E-BP1^{S65} (#9451). Antibodies were diluted in blocking buffer and incubated with membranes overnight at 4°C with gentle rocking. Membranes were washed three to four times for five minutes each in wash buffer (PBS supplemented with 0.1% Tween 20) before adding Anti-Rabbit IgG, HRP-Linked Antibody (Cell Signaling Technology, #7074P2) for one hour at room temperature in blocking buffer. Membranes were washed three to four times for five minutes each in wash buffer and then developed using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific).

Immunofluorescent staining and confocal microscopy imaging. HeLa cells were seeded onto ibiTreat μslide 8-Well slides (Ibidi) and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 1% Pen-Strep (Gibco) and 10% fetal bovine serum until they reached 80-90% confluency before transfection or immunostaining. Imaging was performed using a Stellaris 8 (Leica) confocal microscope. Quantitative analyses were performed using FIJI (3). For mTOR/LAMP1 co-localization immunostaining, HeLa cells were washed once with PBS before

fixation for 15 minutes at room temperature in 4% paraformaldehyde. Cells were washed three times in PBS and then permeabilized with 0.2% Triton X-100 for 10 minutes followed by three washes in PBS. Blocking was performed by adding 3% BSA, 0.2% Triton X-100 for 1 hour at room temperature. sAB-R3E9 and anti-LAMP1 (Cell Signaling Technology, #9091T) antibodies were diluted in 1% BSA, 0.2% Triton X-100 and added for 2 hours at 4°C. Cells were washed three times with PBS. Goat Anti-Human IgG, $F(ab')_2$ fragment specific conjugated with Alexa647 and Goat Anti-Rabbit IgG (H+L) conjugated with Alexa488 (Jackson ImmunoResearch) were diluted in 1% BSA, 0.2% Triton X-100 and added for 45 minutes at room temperature. Cells were washed three times with PBS and incubated with 1 µg/µL DAPI (Thermo Fisher Scientific) for 10 minutes before washing again three times with PBS. PBS in 50% glycerol was added as the final step. For p-$S6^{S240/244}$ (Cell Signaling Technology, #5364), p-4E-$BP1^{T37/46}$ (Cell Signaling Technology, #2855), and mTOR (Cell Signaling Technology, #2983) immunostainings, all steps were performed the same as above with the following exceptions: (i) HeLa cells were first transfected according to the manufacturers protocol, (ii) p-$S6^{S240/244}$, p-4E-$BP1^{T37/46}$, or mTOR Rabbit IgGs were added overnight at 4°C, and (iii) Goat Anti-Rabbit IgG (H+L) conjugated with Alexa647 (Jackson ImmunoResearch) was used for secondary detection.

SI References

1. T. Slezak, *et al.*, An engineered ultra-high affinity Fab-Protein G pair enables a modular antibody platform with multifunctional capability. *Protein Sci* **29**, 141–156 (2020).

2. M. Baidya, *et al.*, Genetically encoded intrabody sensors report the interaction and trafficking of β-arrestin 1 upon activation of G-protein–coupled receptors. *Journal of Biological Chemistry* **295**, 10153–10167 (2020).

3. J. Schindelin, *et al.*, Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**, 676–682 (2012).

SI Figures

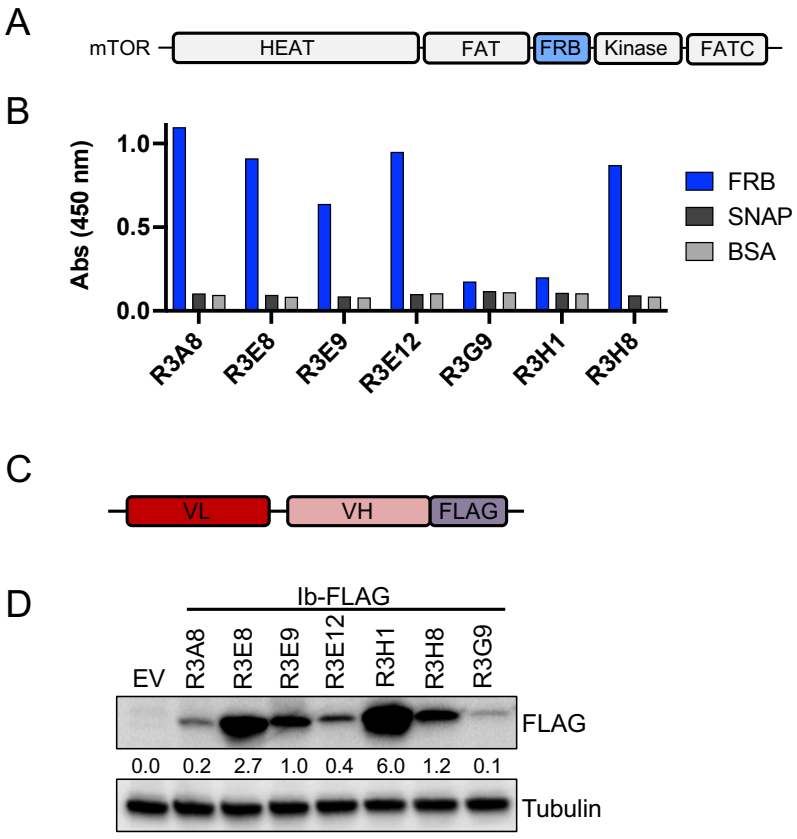

Fig. S1. Selection of synthetic antibody fragments and conversion to single-chain variable fragment intrabodies. (A) Domain map of mTOR with FRB highlighted as the target used for epitope-directed phage display. (B) Representative phage ELISA screening for unique clones resulting from five rounds of phage display biopanning. (C) Construct design for single-chain variable fragment intrabodies with C-terminal FLAG tag. (D) Expi293F cells were transfected with the indicated intrabodies for 48 hours before harvesting for western blot analysis. Values indicate relative intrabody expression levels normalized to R3E9.

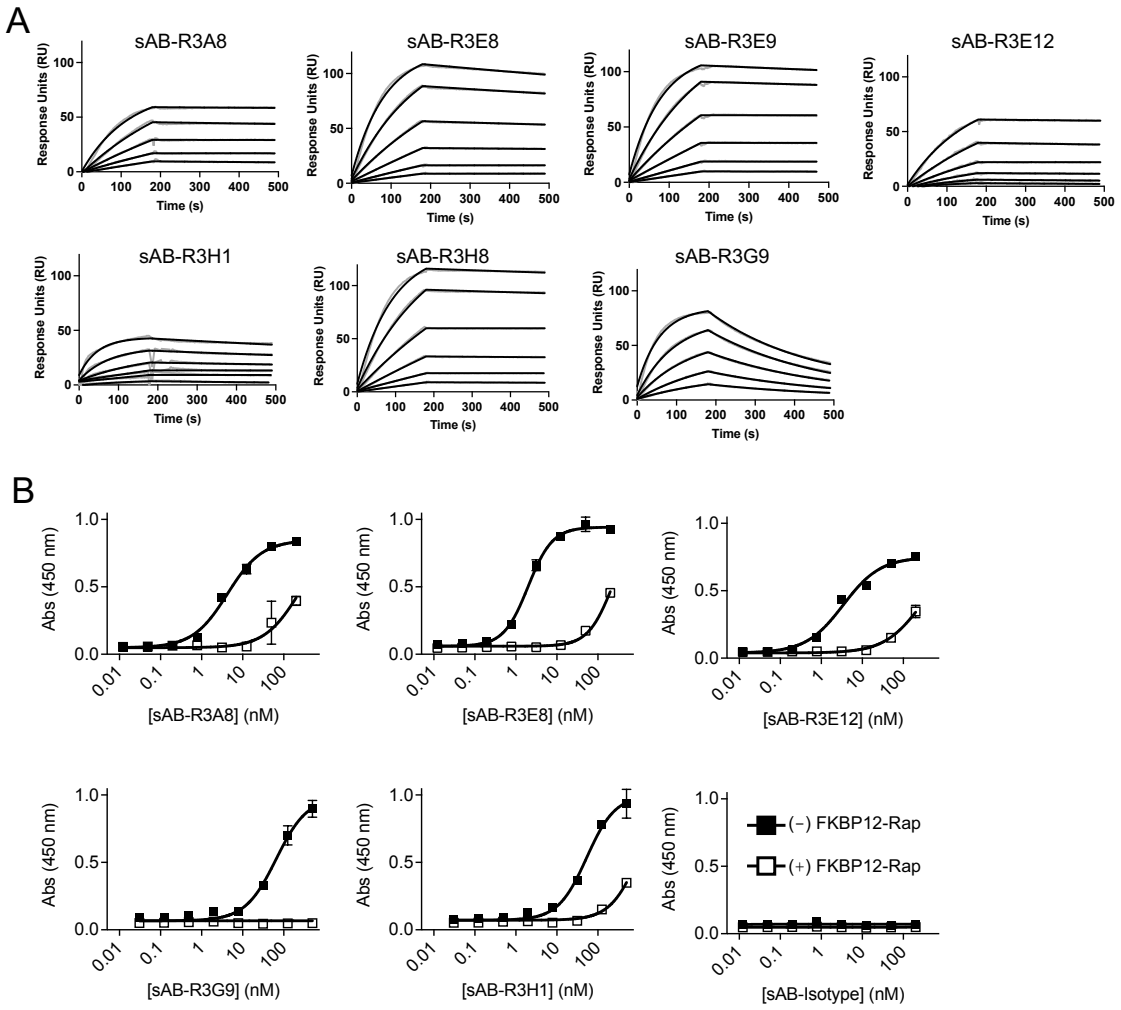

Fig. S2. Biophysical characterization of sABs. (A) Multi-point SPR kinetics for sAB binding to immobilized mTOR^{FRB}. sABs were run as analytes in two-fold serial dilutions. Curve fitting was performed using a 1:1 binding model. (B) Multi-point competitive ELISA screening for mTOR^{FRB} alone (black) or with 10 μM FKBP12-Rapamycin (white) in solution for the indicated sABs (n = 3, mean ± SD).

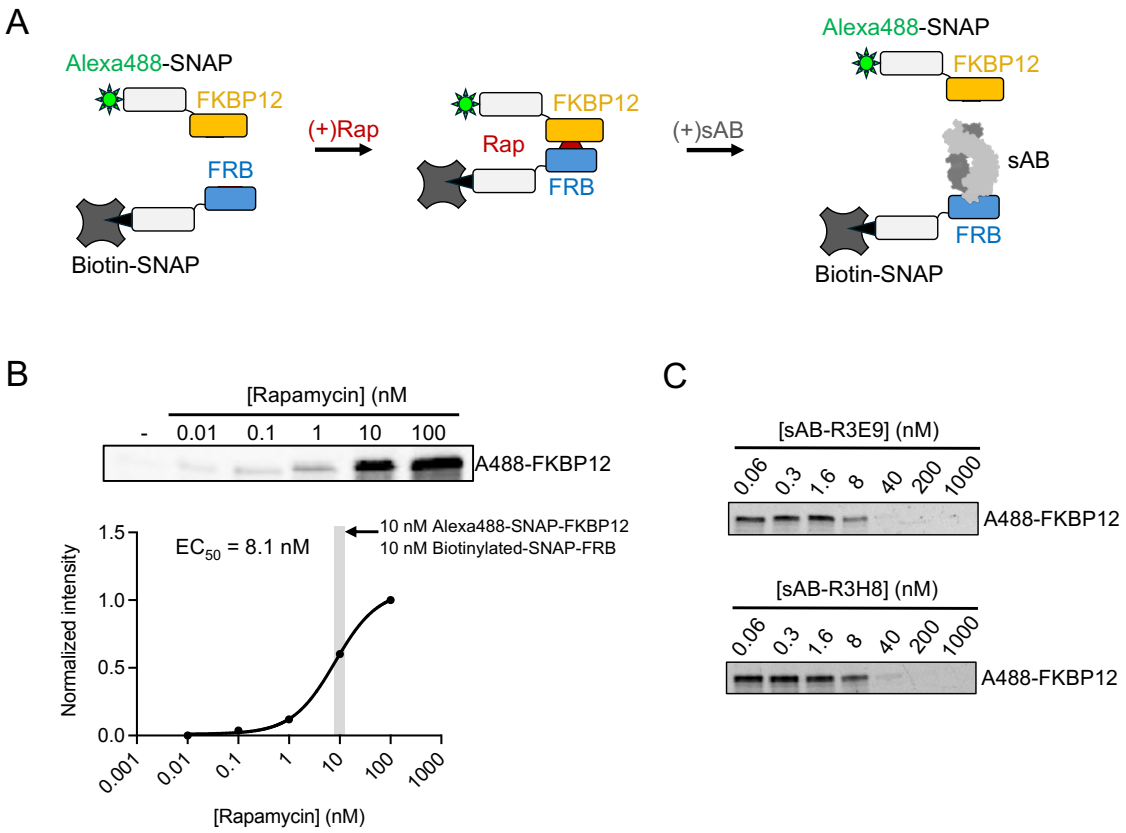

Fig. S3. In vitro pulldown assay for competition of sABs with FKBP12-Rapamycin. (A) Schematic of the fluorescence-based in vitro streptavidin bead pulldown assay. (B) Representative assessment of the assay using 10 nM biotinylated SNAP-mTOR^{FRB} and 10 nM Alexa488-SNAP-FKBP12 incubated with the indicated concentrations of rapamycin. (C) Representative SDS-PAGE readouts for Alexa488 fluorescence from in vitro pulldowns using titrations of sAB-R3E9 (top) and sAB-R3H8 (bottom).

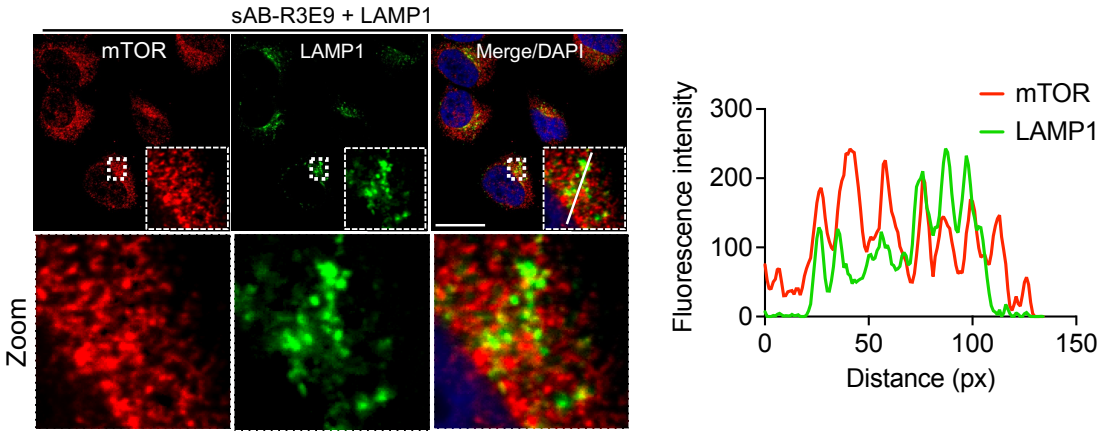

Fig. S4. *Left:* Immunofluorescent co-staining of HeLa cells with sAB-R3E9 (red) and an anti-LAMP1 (green) antibody. Scale bar denotes 20.5 μm. *Right*: Line trace profile from the line drawn in the merged image.

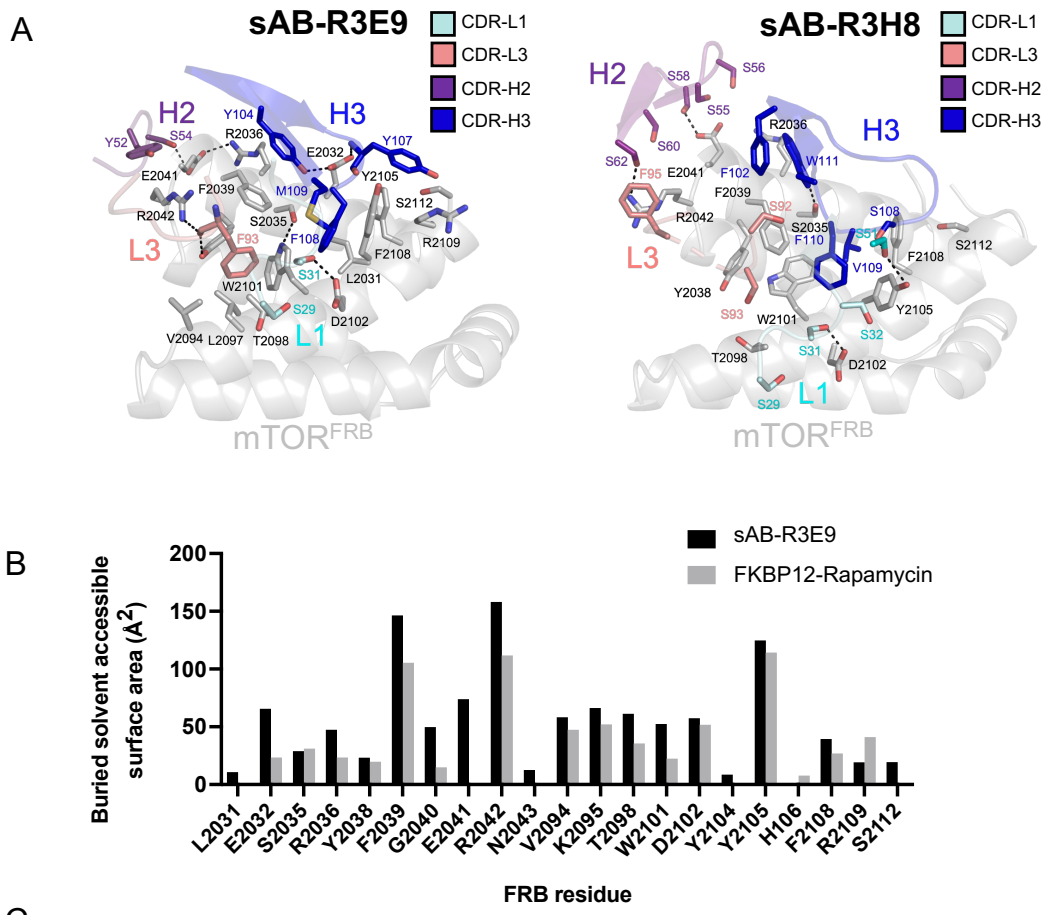

Fig. S5. Detailed analysis of interactions at the mTOR^{FRB} substrate recruitment site. (A) CDR interactions of sAB-R3E9 (left) and sAB-R3H8 (right) with mTOR^{FRB}. (B) Analysis of mTOR^{FRB} residue solvent accessible surface area burial by sAB-R3E9 compared to a previously published structure of mTOR^{FRB} bound by FKBP12-Rapamycin (PDB: 1FAP). (C) Multiple sequence alignment of mTOR and contacts made by the indicated molecules above. Black circles represent interaction of the indicated molecule with *H. sapiens* mTOR^{FRB}. Uniprot IDs from top to bottom: P42345, Q9JLN9, Q9VK45, Q95Q95, P35169, P32600.

A

sAB-R3E9	EC_{50} (nM)
WT	7.8
F93A	12.3
Y104A	89.8
Y107A	9.5
F108A	115.1
M109A	112.0

B

sAB-R3H8	EC_{50} (nM)
WT	5.7
V109A	37.3
F110A	n.d.
W111A	84.2

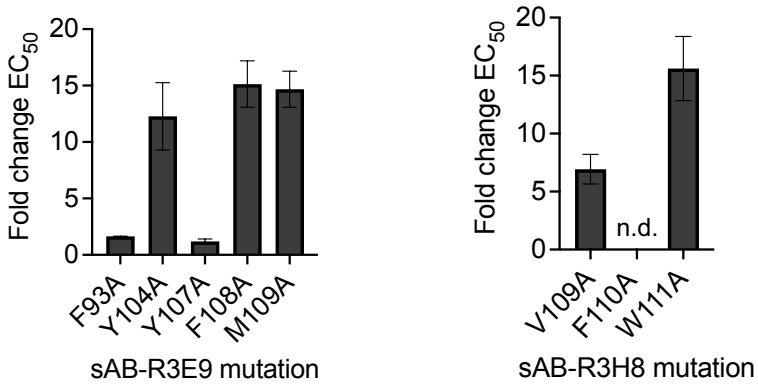

Fig. S6. Alanine scanning mutagenesis of consensus hydrophobic interaction sites within the $mTOR^{FRB}$ substrate recruitment interface. (A) *Top:* EC_{50} values (nM) for alanine mutations made in sAB-R3E9. *Bottom:* Visualization of the fold change in EC_{50} values for sAB-R3E9 alanine mutations (n = 3 independent experiments, mean ± SD). (B) *Top:* EC_{50} values (nM) for alanine mutations made in sAB-R3H8. *Bottom:* Visualization of the fold change in EC_{50} values for sAB-R3H8 alanine mutations. F110A EC_{50} value could not be determined (n.d.) (n = 3 independent experiments, mean ± SD).

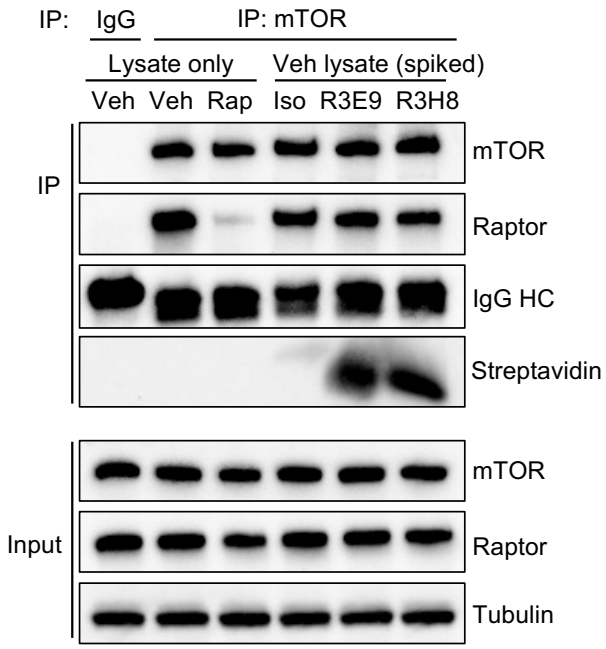

Fig. S7. Representative pan-mTOR co-immunoprecipitation western blot analysis from Expi293F cell lysates alone or lysates spiked with biotinylated sABs. Briefly, cells were treated with vehicle or 200 nM rapamycin for 24 hours. Vehicle treated lysate was split and spiked with biotinylated isotype control (Iso), biotinylated sAB-R3E9, or biotinylated sAB-R3H8 and incubated at 4C before performing pan-mTOR immunoprecipitation for western blot analysis.

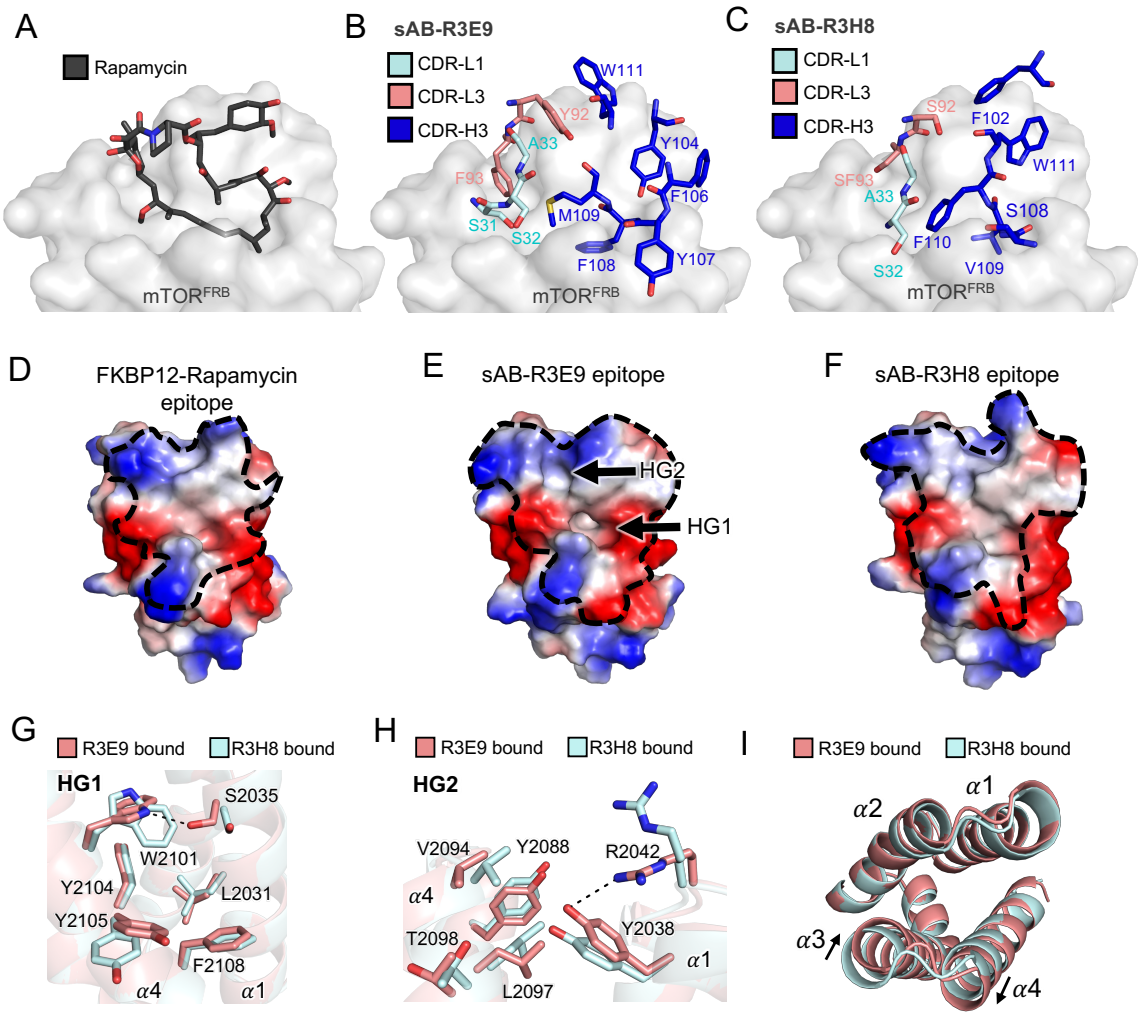

Fig. S8. Structural comparison of sAB-R3E9, sAB-R3H8, and rapamycin. (A) Binding pose of rapamycin from the previously determined FKBP12-Rapamycin-mTOR^{FRB} crystal structure (PDB: 1FAP). (B) sAB-R3E9 residues with similar spatial arrangement as rapamycin. (B) sAB-R3H8 residues with similar spatial arrangement as rapamycin. (D) mTOR^{FRB} surface electrostatics when bound to FKBP12-Rapamycin (PDB: 1FAP). (E) mTOR^{FRB} surface electrostatics when bound to sAB-R3E9. Hydrophobic grooves 1 and 2 (HG1 and HG2) marked by arrows. (F) mTOR^{FRB} surface electrostatics when bound to sAB-R3H8. Epitopes of the indicated molecules are traced with black dashed line. (G) Visualization of side chains in mTOR^{FRB} that form HG1. (H) Visualization of side chains in mTOR^{FRB} that form HG2. (I) Conformational change of mTOR^{FRB} in α3 and α4 helices in the R3E9 bound state (salmon) compared to R3H8 bound state (pale cyan).

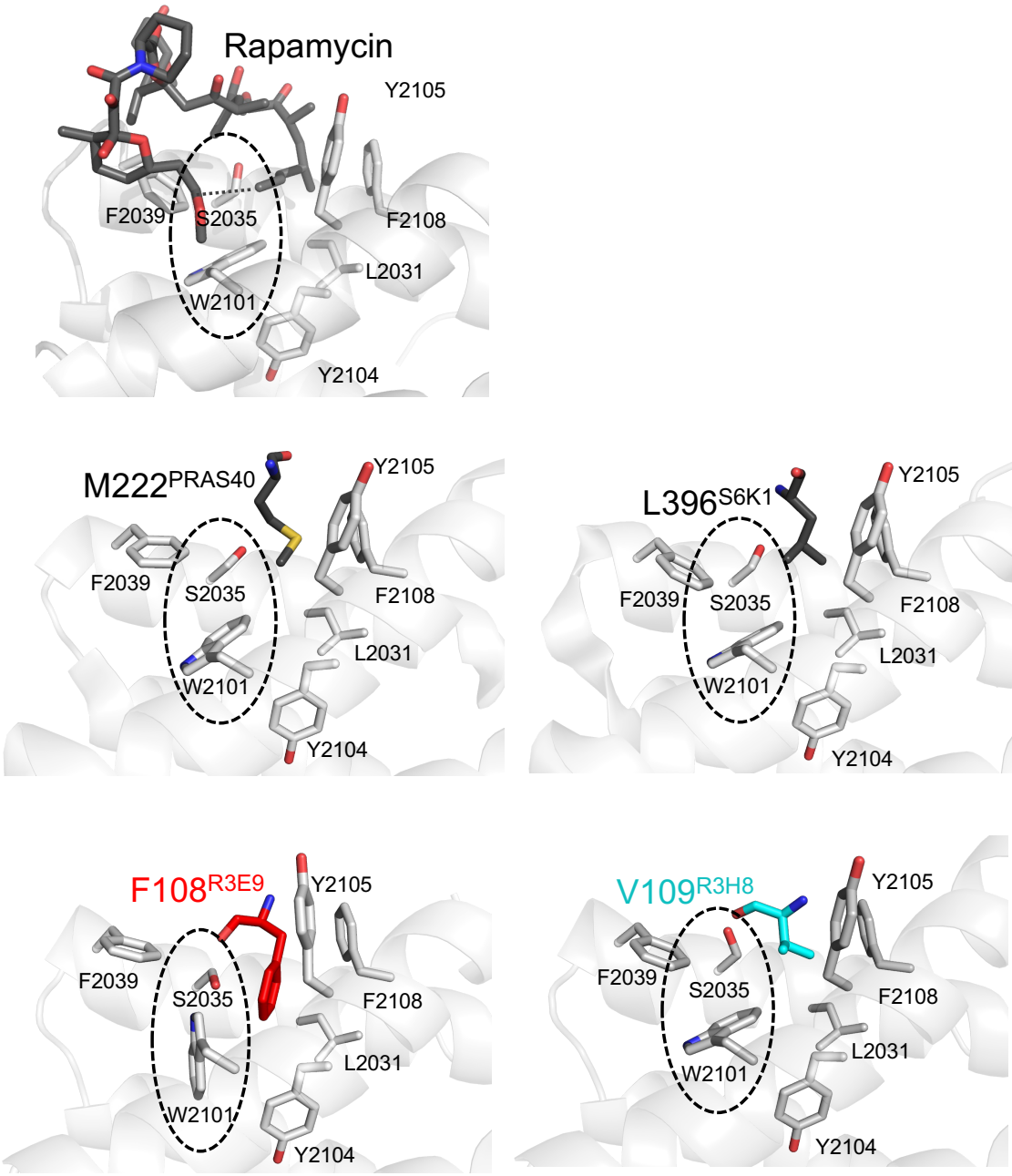

Fig. S9. View of W2101 and S2035 (circled) interactions in hydrophobic groove 1 (HG1) from previously published structures of mTOR^{FRB} bound by FKBP12-Rapamycin (PDB: 1FAP; some rapamycin atoms omitted for clarity of S2035 represented by dashed line), S6K1 (PDB: 5WBH), and PRAS40 (PDB: 5WBU). These interactions are compared to sAB-R3E9 bound and sAB-R3H8 bound structures, illustrating the novel conformation of mTOR^{FRB} stabilized by sAB-R3E9.

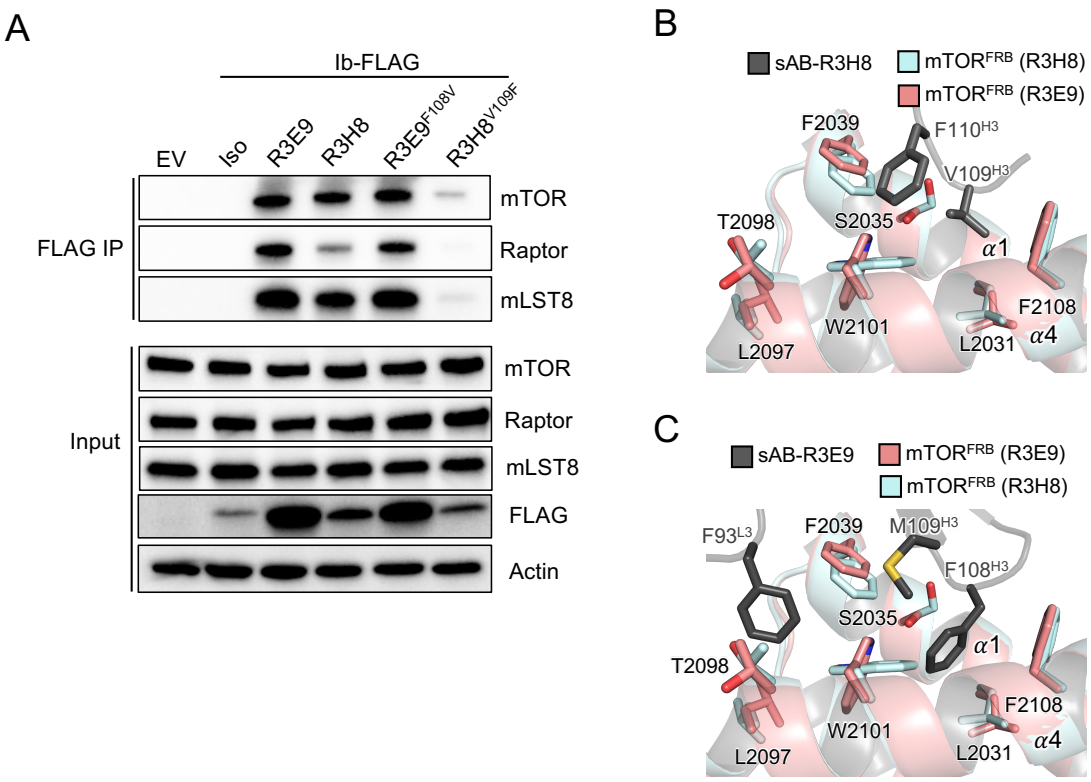

Fig. S10. Effects of side chain stereochemistry in Ib-R3E9 and Ib-R3H8 variants. (A) Representative intrabody immunoprecipitation-western blot analysis of Expi293F cells transfected with empty vector (EV), isotype control (Iso), R3E9, or R3H8 FLAG-tagged intrabodies (Ib-FLAG) for 48 hours. (B) Alignment of $mTOR^{FRB}$ structures with important sAB-R3H8 paratope residues shown in grey. (C) Alignment of $mTOR^{FRB}$ structures with important sAB-R3E9 paratope residues shown in grey. This demonstrates that coordination of $F108^{H3}$ is incompatible with the conformation of W2101 in the sAB-R3H8 bound structure of $mTOR^{FRB}$.

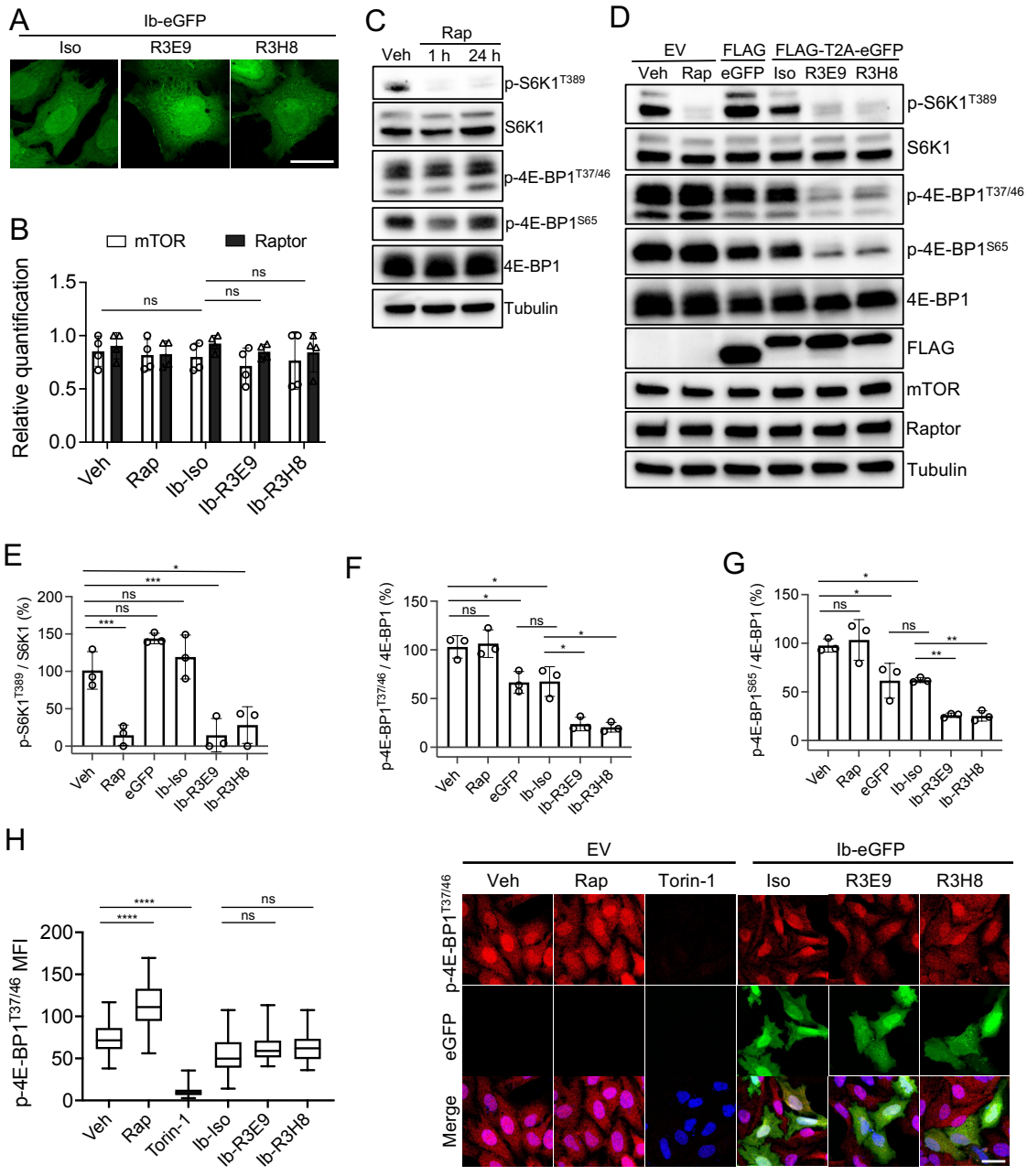

Fig. S11. Characterization of genetically encoded intrabodies. (A) Confocal microscopy visualization of eGFP-tagged intrabodies transfected into HeLa cells for 24 hours. Scale bar denotes 32.3 μm. (B) Relative quantification of total mTOR and Raptor protein expression from eGFP(+) Expi293F cells isolated by FACS used in the western blot in Fig. 4B (n = 4 biological replicates, mean ± SD. ns = not significant, one-way ANOVA). (C) Western blot analysis of mTORC1 substrate phosphorylation after 1 hour and 24 hours of 100 nM rapamycin treatment in Expi293F cells. (D) Representative western blot analysis of Expi293F cells transfected with empty vector (EV), FLAG-eGFP, or isotype control (Iso), R3E9, and R3H8 FLAG-T2A-eGFP tagged intrabodies (Ib-FLAG-T2A-eGFP) for 24 hours before isolation of eGFP(+) cells by FACS. Vehicle and 100 nM rapamycin treatment was 16 hours. (E-G) Quantification of the experiment in A for the indicated phosphorylation levels (n = 3 biological replicates, mean ± SD. ns = not significant, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, one-way ANOVA). (H) *Left:* Quantification of immunostained p-4E-BP1$^{T37/46}$ levels in HeLa cells treated with vehicle, 250 nM rapamycin, 250 nM Torin-1, or the

indicated eGFP-tagged intrabodies (n = 32, 33, 29, 38, 28 and 29 cells. ns = not significant, ****$P \leq 0.0001$, one-way ANOVA with Tukey-Kramer test). *Right:* Representative immunofluorescent confocal microscopy analysis of eGFP-tagged intrabody expression for 24 hours and of p-4E-BP1$^{T37/46}$ levels in HeLa cells. Scale bar denotes 32.3 μm. (n = 2 independent experiments).

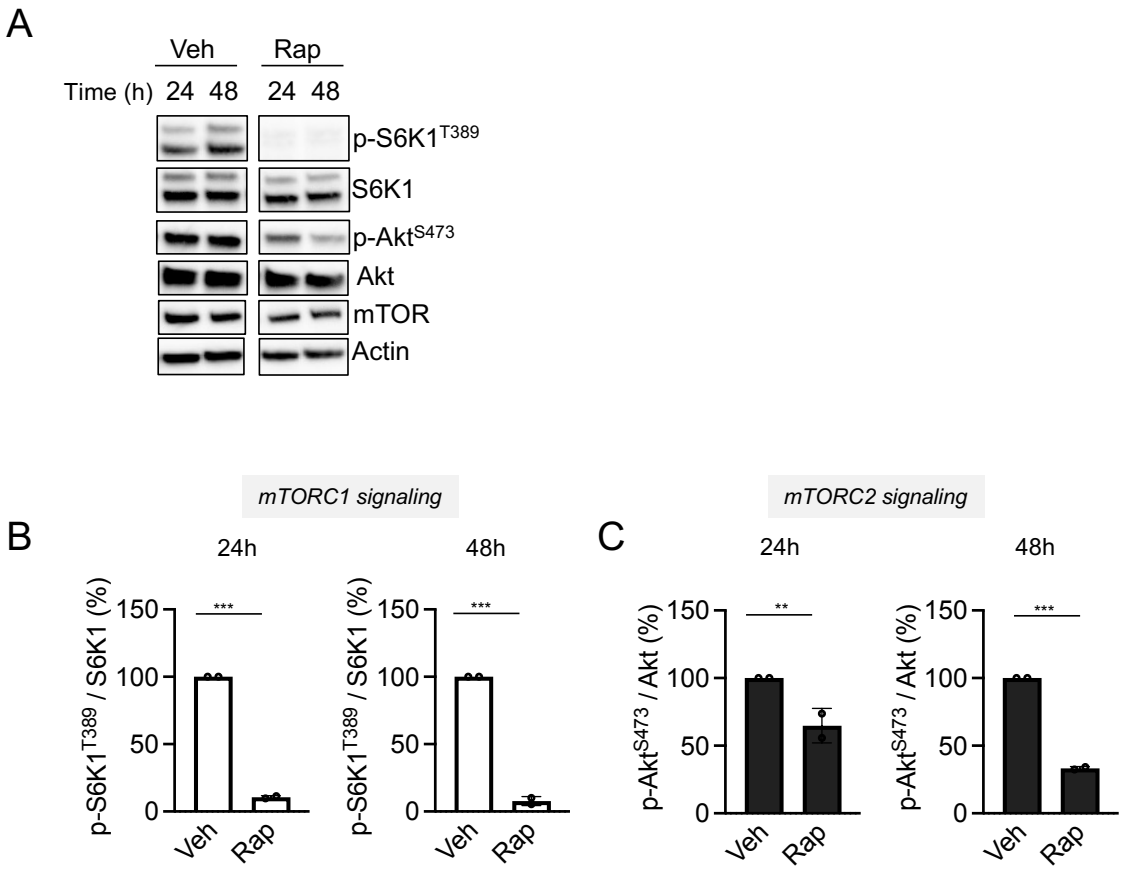

Fig. S12. Comparison of intrabody-based mTOR modulation to rapamycin. (A) Representative western blot analysis of Expi293F cells treated with vehicle or 100 nM rapamycin for the indicated time points. The western blots were cropped and rearranged from the original images to remove irrelevant samples other than vehicle and rapamycin treatments for 24 or 48 hours. (B) Quantification of p-S6K1^{T389} phosphorylation from C. (n = 2 biological replicates, mean ± SD. ***$P \leq 0.001$, one-way ANOVA). (C) Quantification of p-Akt^{S473} phosphorylation from C. (n = 2 biological replicates, mean ± SD. **$P \leq 0.01$, ***$P \leq 0.001$, one-way ANOVA).

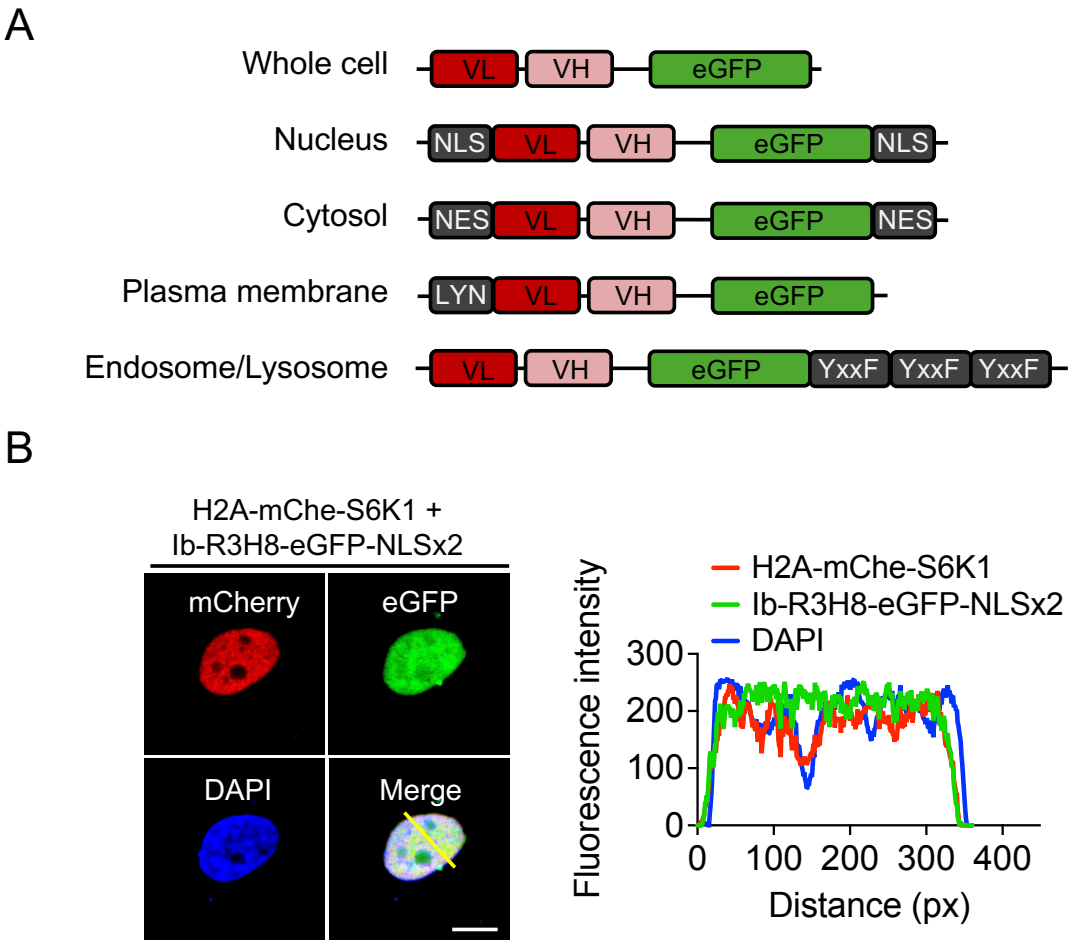

Fig. S13. Directing the expression of intrabodies to selected subcellular locations. (A) Schematic of eGFP-tagged intrabody constructs bearing the indicated subcellular localization tags. (B) *Left:* Representative confocal microscopy analysis of the nuclear colocalization of H2A-mCherry-S6K1 (red) and Ib-R3H8-eGFP-NLSx2 (green) in HeLa cells. Scale bar denotes 9.2 μm. *Right:* Line trace quantification of fluorescence intensity along the yellow line drawn in the merged image.

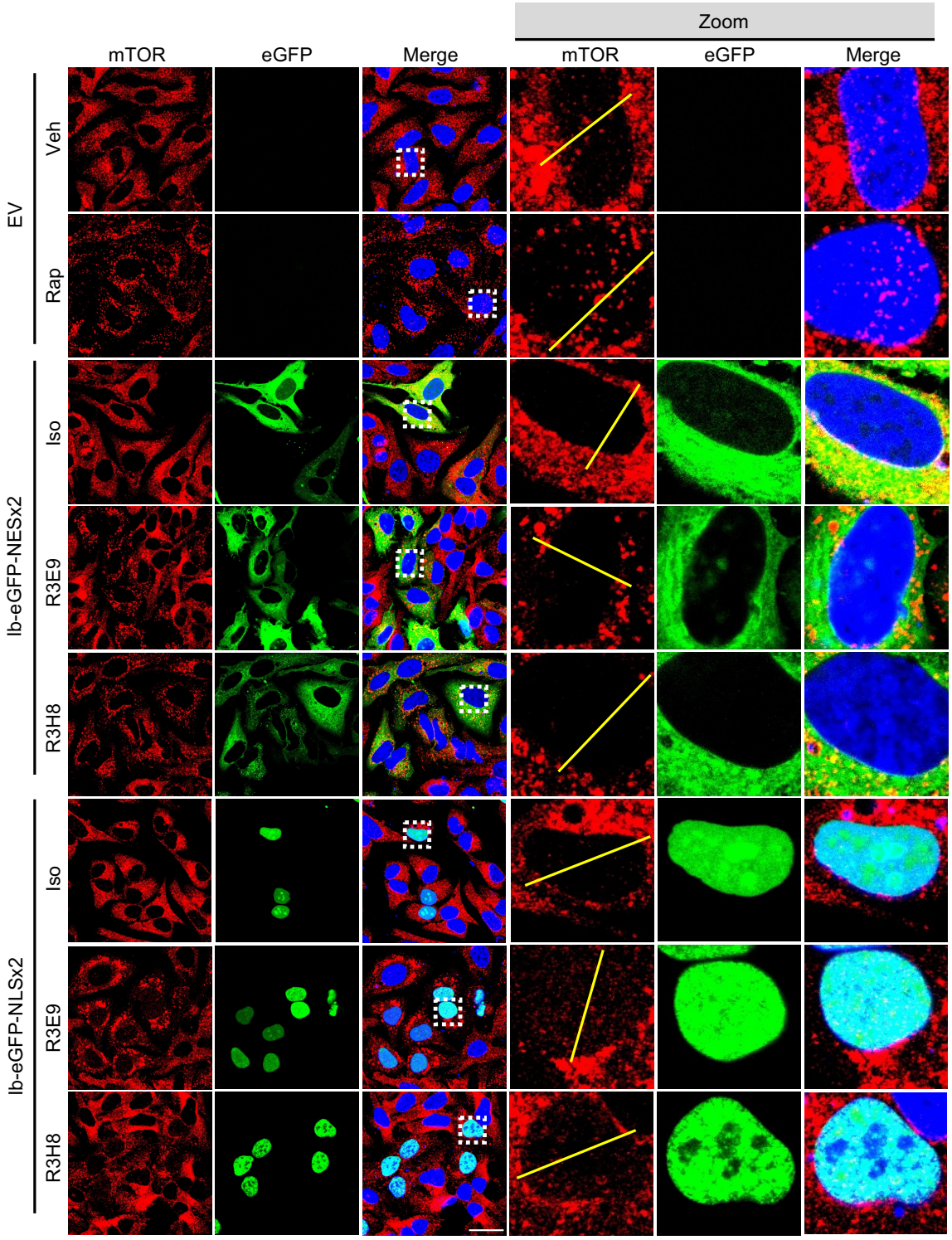

Fig. S14. Confocal microscopy analysis of mTOR localization in cells expressing cytosol- and nucleus-restricted eGFP-tagged intrabodies. HeLa cells were transfected with the indicated intrabody constructs for 24 hours and then immunostained with an anti-mTOR antibody. Scale bar denotes 32.3 μm. (n = 2 independent experiments).

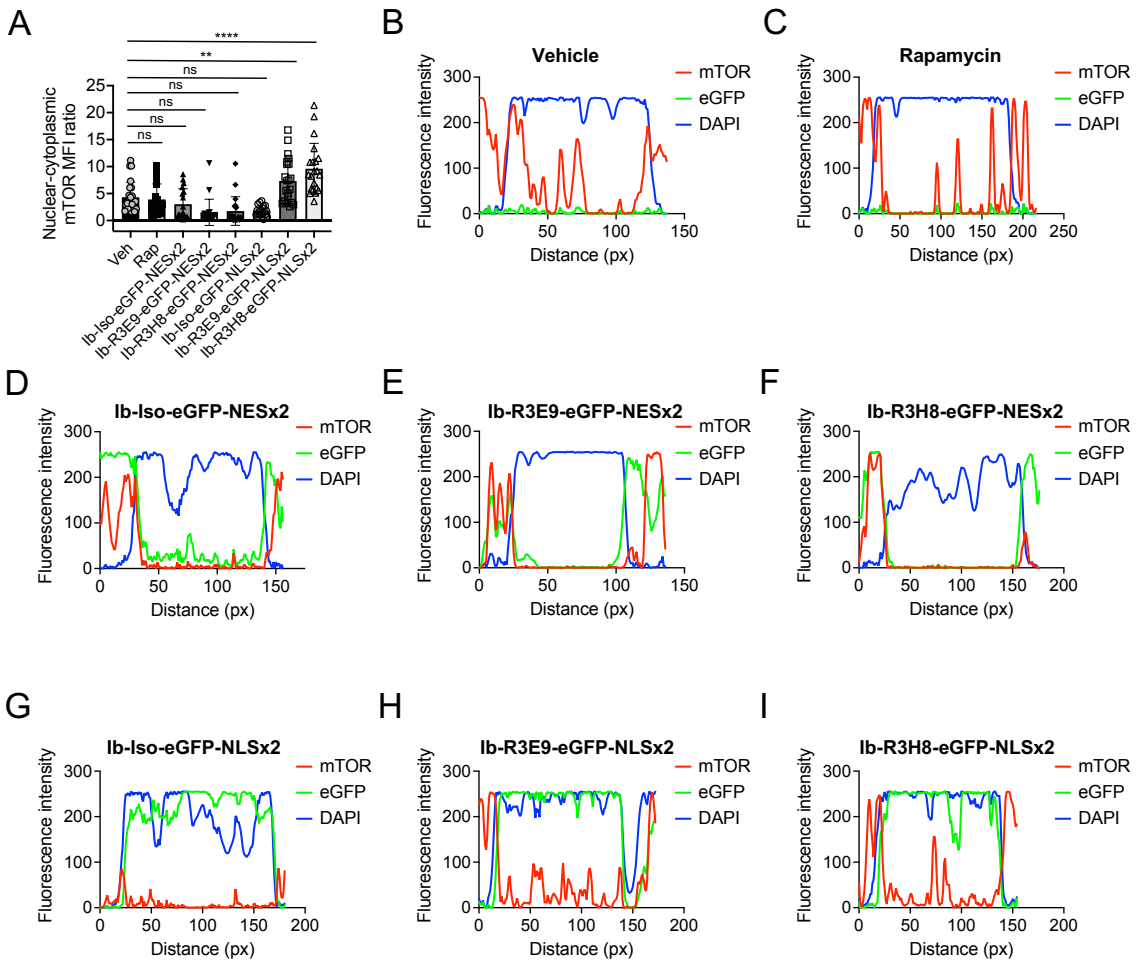

Fig. S15. Quantification of mTOR localization in cells expressing cytosol- and nucleus-restricted eGFP-tagged intrabodies. (A) Nuclear-cytoplasmic ratio of mean fluorescence intensity (MFI) for mTOR in HeLa cells expressing the indicated constructs (n = 23, 16, 18, 20, 20, 18, 23, and 21 cells from two independent experiments. ns = not significant, $^{**}P \leq 0.01$, $^{****}P \leq 0.0001$, one-way ANOVA with Tukey-Kramer test). (B) Line trace profile from the yellow line shown in Supplementary Fig S14 for vehicle treated cells, (C) rapamycin treated cells, (D) Ib-Iso-eGFP-NESx2, (E) Ib-R3E9-eGFP-NESx2, (F) Ib-R3H8-eGFP-NESx2, (G) Ib-Iso-eGFP-NLSx2, (H) Ib-R3E9-eGFP-NLSx2, and (I) Ib-R3H8-eGFP-NLSx2.

Table S1. X-ray diffraction data collection and refinement statistics.

	sAB-R3H8•mTOR^{FRB} (PDB 9DL0)	sAB-R3E9•mTOR^{FRB} (PDB 9DBO)
Wavelength (Å)	1.0332	1.0332
Space group	*P 21 2 21*	*P 1 2 1*
Cell dimensions		
a, b, c (Å)	87.613, 110.042, 131.079,	45.055, 69.732, 89.413
α, β, γ (°)	90, 90, 90	90, 90.12, 90
Resolution (Å)	56.31 - 2.0 (2.02 - 2.0)*	45.05 - 1.55 (1.57 - 1.55)
R_{merge}	0.054 (0.955)	0.056 (0.671)
R_{meas}	0.076 (1.351)	0.079 (0.949)
R_{pim}	0.054 (0.955)	0.056 (0.671)
$CC_{1/2}$	0.998 (0.295)	0.994 (0.475)
$I / \sigma I$	8.8 (1.0)	6.8 (1.0)
Completeness (%)	94.32 (99.75)	93.9 (71.0)
Redundancy	2.0 (2.0)	1.9 (1.8)
No. reflections	81,284	75,225
R_{work} / R_{free}	0.2050 / 0.2353	0.2043 / 0.2338
No. atoms		
Protein	8113	4099
Solvent	373	548
Protein residues	1056	529
R.M.S deviations		
Bond lengths (Å)	0.008	0.008
Bond angles (°)	0.99	1.1
Ramachandran favored (%)	97.41	97.9
Allowed (%)	2.59	2.1
Outliers (%)	0	0
Rotamer outliers (%)	1.22	0.44
Clashscore	2.71	1.98
Average B-factor		
Protein	46.18	14.66
Solvent	45.02	34.05

*Values in parentheses are for the highest resolution shell