

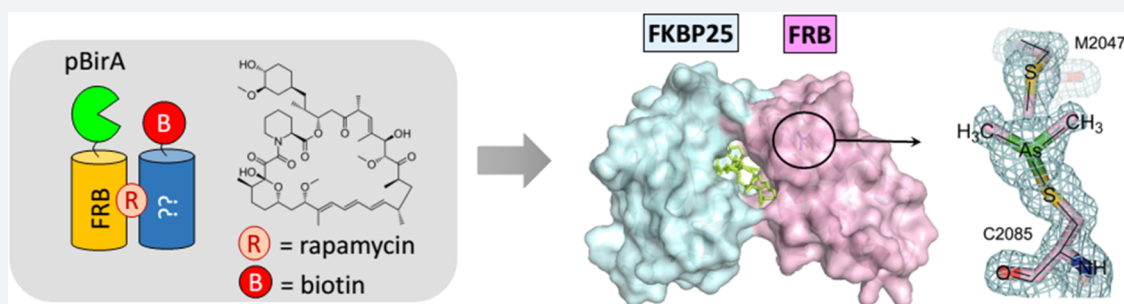
Proximity-Directed Labeling Reveals a New Rapamycin-Induced Heterodimer of FKBP25 and FRB in Live Cells

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S Supporting Information



ABSTRACT: Mammalian target of rapamycin (mTOR) signaling is a core pathway in cellular metabolism, and control of the mTOR pathway by rapamycin shows potential for the treatment of metabolic diseases. In this study, we employed a new proximity biotin-labeling method using promiscuous biotin ligase (pBirA) to identify unknown elements in the rapamycin-induced interactome on the FK506-rapamycin binding (FRB) domain in living cells. FKBP25 showed the strongest biotin labeling by FRB–pBirA in the presence of rapamycin. Immunoprecipitation and immunofluorescence experiments confirmed that endogenous FKBP25 has a rapamycin-induced physical interaction with the FRB domain. Furthermore, the crystal structure of the ternary complex of FRB–rapamycin–FKBP25 was determined at 1.67-Å resolution. In this crystal structure we found that the conformational changes of FRB generate a hole where there is a methionine-rich space, and covalent metalloid coordination was observed at C2085 of FRB located at the bottom of the hole. Our results imply that FKBP25 might have a unique physiological role related to metallomics in mTOR signaling.

INTRODUCTION

Recently, proximity-directed labeling methods^{1–3} have been developed based on enzymatic reactions that are mediated by engineered ascorbate peroxidase^{4–6} or promiscuous biotin ligase (pBirA).^{7–12} In the pBirA method, an *in situ* reactive biotin-activated ester can be generated in living cells, such as biotin-adenosine-5'-monophosphoester (biotin-AMP), that preferentially reacts with the lysine residues of neighboring proteins. Because this labeling occurs via covalent bond formation, the labeled proteins can be isolated by standard pull-down methods using streptavidin (SA) beads and are then readily identified by mass spectrometry. Using these methods, collectively referred to as BioID, many interesting subcompartmental-localized proteomes such as the nuclear envelope membrane proteome,⁷ nuclear pore complex,^{8,13} centrosome,¹⁰ centrosome–cilium interface,¹¹ and cadherin complex¹² have been recently identified.

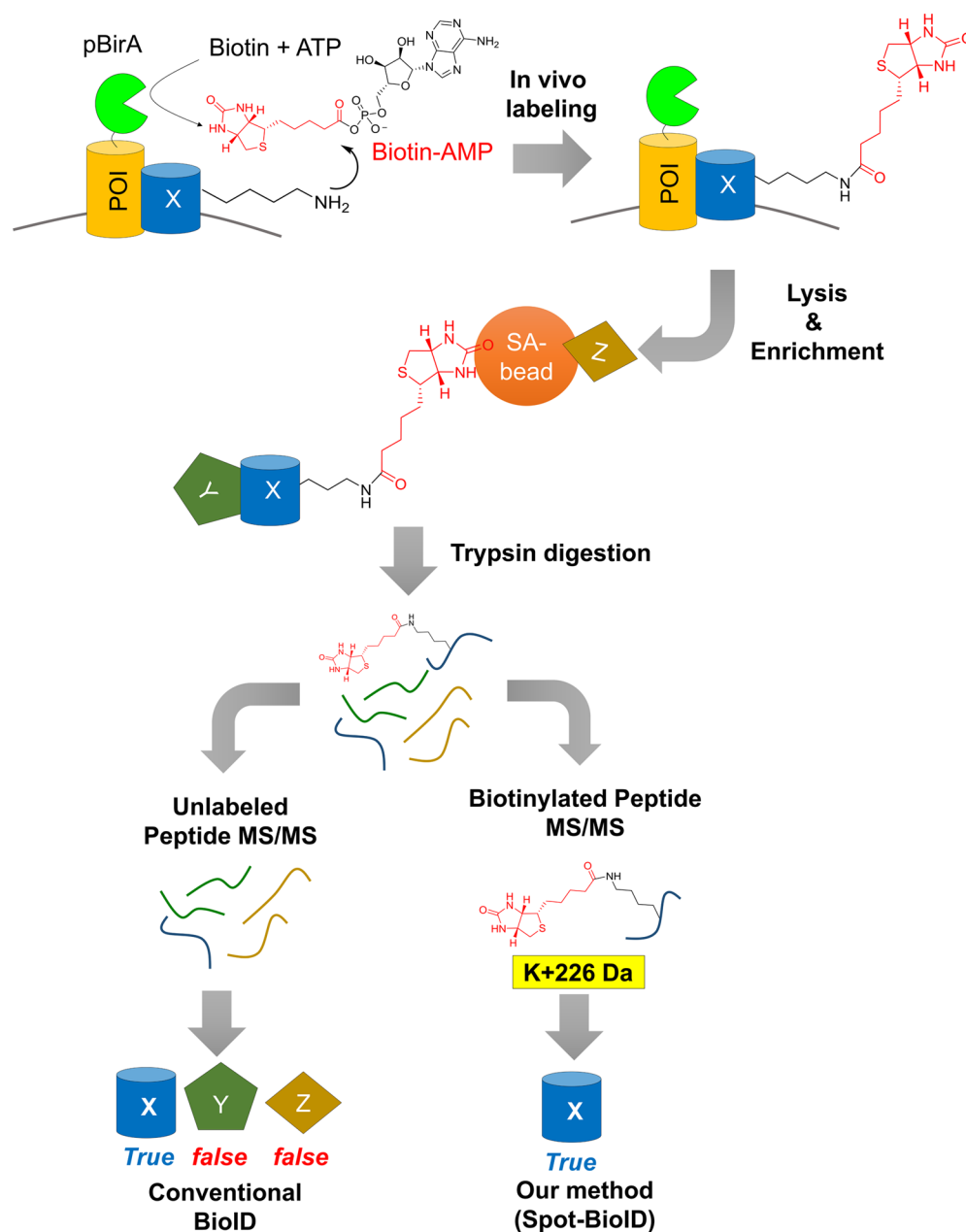
In these methods, the mass identification of biotin-labeled proteins is usually performed by detection of unlabeled peptides from labeled proteins after proteolysis and enrichment, because the population of unlabeled peptides is more

abundant than the labeled peptide population. To discriminate between unlabeled peptides following a labeling event (Protein X and Y in Scheme 1) and nonspecific bead-binding proteins (Protein Z in Scheme 1), unlabeled peptides can be tagged with a different isotope composition [e.g., SILAC^{4,5} or iTRAQ⁶] during the sample preparation, and then peptides from a labeling event can be identified according to the expected isotopic ratio. However, this identification method based on unlabeled peptides has a caveat. As shown in Scheme 1, if an unlabeled protein (Protein Y) has strong binding affinity toward a labeled protein (Protein X) and can survive on the bead during the washing step, the identified peptides from this unlabeled protein (Protein Y) could be mistakenly regarded as a “labeled protein.” Therefore, the most direct method to identify a proximity-labeled protein is to identify a biotin-labeled peptide after enrichment; however, no such method has been reported to date. Thus, we tested whether our new proximity-labeled protein ID method (Spot-BioID) could

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Scheme 1. MS Analysis of Biotin-Labeled Proteins Produced by Proximity Labeling



identify the rapamycin-induced interacting protein of the FK506-rapamycin binding (FRB) domain of mammalian target of rapamycin (mTOR).

Rapamycin is a macrocyclic small molecule that is synthesized by soil bacteria, including *Streptomyces hygroscopicus*. Rapamycin was first identified as an antifungal agent but has also been shown to possess immunosuppressive¹⁴ and anticancer activity.¹⁵ Recently, rapamycin treatment was also shown to prevent pathological protein aggregation in neurological disorders by recruiting autophagy activity and could inhibit epileptic seizures in mice.¹⁶ Rapamycin treatment was also shown to be effective in prolonging the lifespans of various experimental models, including mice.¹⁷

In mammals, several FK506-binding proteins (FKBPs) have been identified as rapamycin-binding proteins,¹⁸ including FKBP12, which forms a heterodimer complex with mTOR and allosterically inhibits its activity.¹⁹ Because mTOR signaling

is a core pathway to regulate cell growth and cellular metabolism, controlling the mTOR pathway by rapamycin has been proposed as a potentially useful treatment in diverse human diseases such as cancer, diabetes, obesity, neurological diseases, and genetic disorders.²⁰

mTOR is a large protein (288 kDa), and its small domain, the FRB domain (11 kDa, amino acids 2021–2113), is responsible for FKBP12–rapamycin binding.¹⁹ The crystal structure of the ternary complex of FRB–rapamycin–FKBP12 revealed that FRB and FKBP12 occupy two different hydrophobic faces of rapamycin simultaneously.²¹ Because protein–protein interactions between FRB and FKBP12 only occur in the presence of rapamycin, many biotechnologies have been developed to achieve spatiotemporal control of protein translocation^{22–25} and enzyme activity.^{26–32} In addition, this complex also provides a model system for studying

spatiotemporal protein–protein interactions for the development of various types of genetically encoded tags.^{33–35}

FKBP12 is currently recognized as the exclusive main protein factor controlling mTOR activity in the presence of rapamycin. However, there are also several other reported FKBP proteins with the potential to have rapamycin-induced interactions with mTOR.^{36,37} Thus, in this study, we tested the ability of our new proximity labeling ID method to determine whether FKBP12 is the only major interactor of FRB, and whether the method could reveal other binding partners that have not been previously characterized by conventional methods.

RESULTS

MS/MS Identification of Proximity-Labeled Proteins.

We conducted proximity labeling experiment with FRB–pBirA in living cells. FRB–pBirA showed a whole-cell expression pattern, and the biotinylation activity of FRB–pBirA seemed promising in both the rapamycin-treated and control cells (Figure 1B). We further assessed whether FRB–pBirA labeled a distinct protein population in a living cell by conducting a Western blot experiment (Figure 1C, see Experimental Section). FRB–pBirA generated numerous biotinylated protein bands, and these band patterns were very similar between the rapamycin-treated sample (FRB-R) and the no rapamycin-treated sample (FRB-NoR) (lanes 1 and 2, Figure 1C). However, we observed one very strongly biotin-labeled protein at around 25 kDa in the SA-HRP pattern of the FRB-R sample (lane 1, Figure 1C), which did not appear in the FRB-NoR sample (lane 2, Figure 1C). We also found that this 25-kDa protein was reproducibly labeled by rapamycin treatment in all stable cell clones of FRB–pBirA constructed in HEK293T-Rex cells (Figure 1D). We postulated that this protein was most likely labeled by interacting with FRB–pBirA in the presence of rapamycin. However, the labeled protein could not be FKBP12 because FKBP12 (12 kDa) could not be detected at 25 kDa in a denaturing SDS–PAGE gel. Thus, we attempted to identify this protein among the labeled peptides using the Spot-BioID workflow (Scheme 1).

To identify biotin-labeled proteins by FRB–pBirA, we performed two independent pBirA-labeling experiments for stably expressed FRB-R and FRB–pBirA in HEK293T-Rex cells. For direct observation of each biotinylated peptide per sample, we expected to observe biotin-attached modification of the lysine residue (Lys + 226 Da), because the primary amine of lysine is a major target of the biotin-AMP ester.⁷ Thus, we designed our workflow to focus on the enrichment of biotinylated peptides via on-bead trypsin digestion after streptavidin-bead enrichment (see Experimental Section). This procedure allowed us to reduce the sample complexity because most of the abundant unlabeled peptides could be washed out at this step.

From the MS/MS-identified biotin-labeled peptides (see Experimental Section), we extracted the “labeled site” information on each labeled peptide, because there were many cases in which the same modified sites were redundantly identified in different partially trypsin-digested peptides. Then, the extracted labeled sites that were reproducibly found within both replicates were finally selected. From this stringent analysis, we extracted a total of 92 unique labeled sites, which could be clustered into three groups (Group I to III) according to the level of overlap between the FRB-R and FRB-NoR samples (Figure 2A).

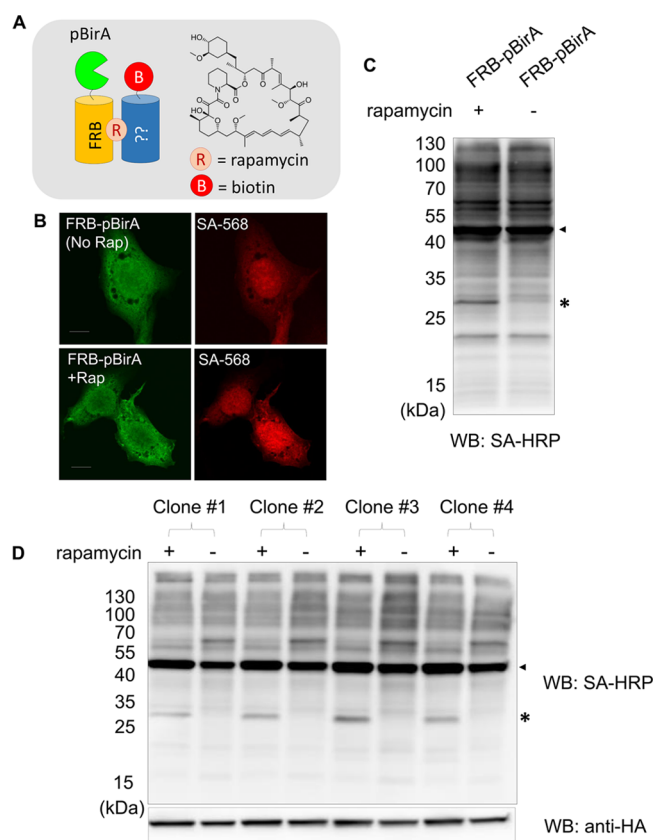


Figure 1. Proximity labeling of FRB–pBirA in living cells. (A) Schematic representation of the proximity labeling reactions of FRB-conjugated promiscuous biotin ligase (FRB–pBirA) in the presence of rapamycin. Circle R represents rapamycin and circle B represents biotin. The chemical structure of rapamycin is shown. (B) Confocal images of the expression of FRB–pBirA and its biotinylation protein pattern with rapamycin or without rapamycin treatment for 16 h in U2OS cells. Scale bar = 10 μ m. (C) Streptavidin (SA)-HRP Western blot of proteins biotinylated by transiently expressed FRB–pBirA with or without rapamycin treatment for 16 h in HEK293T cells. (D) SA-HRP Western blot of biotinylated proteins and anti-HA Western blot of stably expressed FRB–pBirA in different stable clones of HEK293T-Rex cells. Strongly biotinylated protein around 25 kDa is marked with an asterisk, and self-labeled FRB–pBirA (molecular mass = 48.5 kDa) is marked with a black arrow in (C) and (D).

Identification of the Rapamycin-Induced Interactome by Subtraction of Background Labeled Sites.

We found a considerable number of labeled sites that overlapped between the FRB-R and FRB-NoR samples. These overlapping labeled biotinylation sites (Group I) should be regarded as the FRB-interactome. In Group I, a total of 63 biotin-labeled sites were found among 57 identified proteins. This considerable overlap should be related to the similar biotinylated band pattern observed for FRB–pBirA (Figure 1D) regardless of rapamycin treatment.

Interestingly, we also found several proteins, including ECD, FEN1, FKBP25, GSPT1, LARP1, PCNP, and TIPRL, that were exclusively labeled by FRB–pBirA only when the cells were treated with rapamycin (Group II) (Figure 2B). This suggests that the proteins in Group II are highly likely to be rapamycin-induced interaction partners of FRB in living cells. In this group, we found reasonable evidence of associations of LARP1, TIPRL, and FKBP25 with mTOR or rapamycin according to the literature. LARP1 is known to regulate mTOR activity by

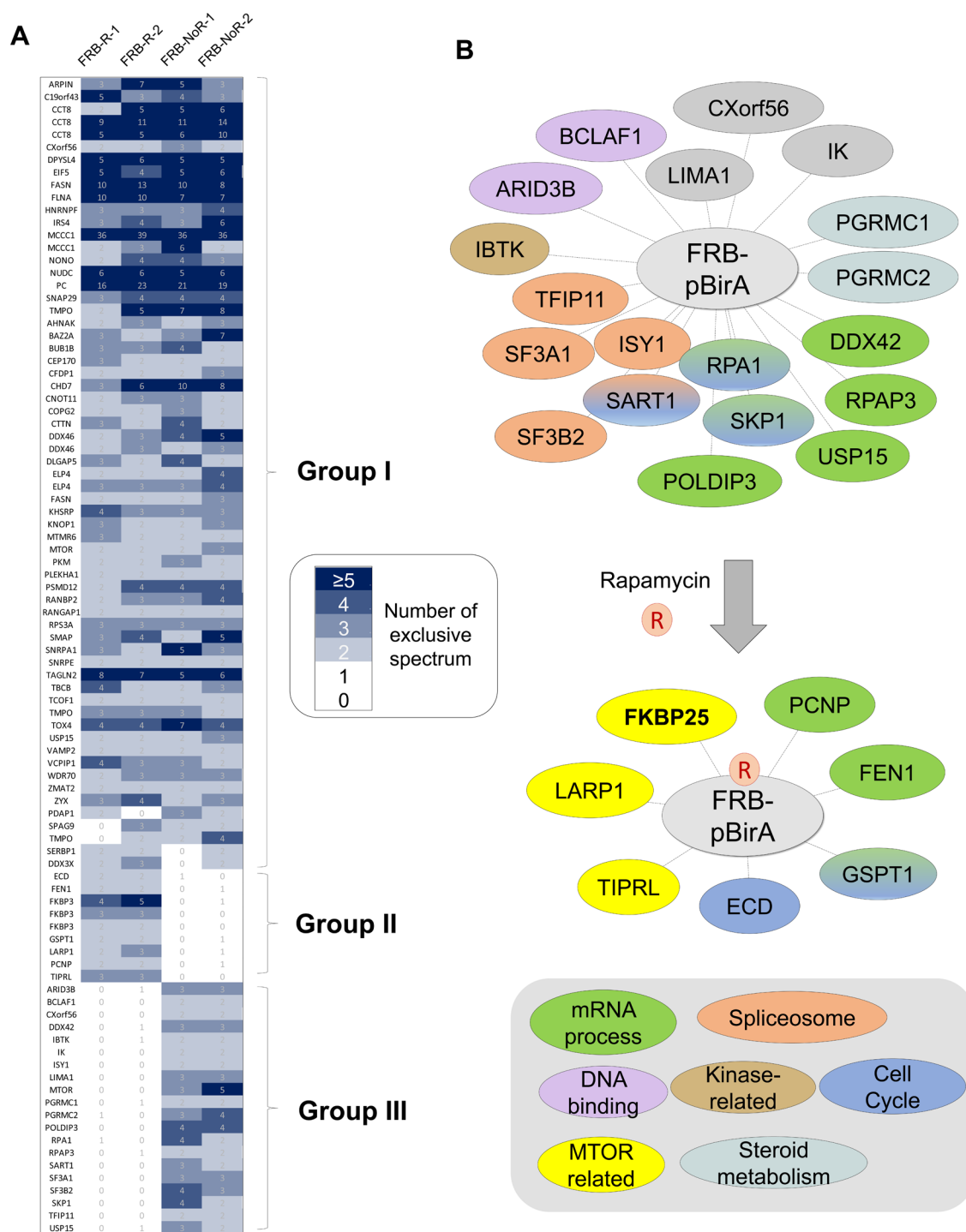


Figure 2. Proteomic analysis of proteins proximity-labeled by FRB–pBirA with or without rapamycin treatment. (A) Overview of 92 sites biotin-labeled by stably expressed FRB–pBirA in HEK293T-Rex cells with or without rapamycin treatment. Color intensity represents the unique spectral counts of each labeled site per biological replicate. Detailed information regarding the identified labeled site for each labeled protein is shown in [Supplementary Data Set 1](#), Supporting Information. (B) Rapamycin-dependent biotin labeling of proteins by FRB–pBirA. Upper protein cluster shows proteins that contain sites exclusively biotin-labeled by FRB–pBirA in the absence of rapamycin (Group III), and lower protein cluster shows proteins that contain sites exclusively biotin-labeled by FRB–pBirA in the presence of rapamycin (Group II). Proteins with previously reported functions are marked by a different color.

post-transcription control, TIPRL is known to act on mTORC1 signaling in conditions of amino acid starvation,³⁸ and FKBP25 is a rapamycin-binding protein.³⁹ It is noteworthy that only FKBP25 (also known as FKBP3) showed multiple biotin-labeled sites (K80, K86, K89) among the Group II proteins ([Figure 3](#)), which indicates that strong proximity-driven

labeling occurred on FKBP25 by FRB–pBirA in the presence of rapamycin.

We also found that some proteins were exclusively labeled by FRB–pBirA in the absence of rapamycin (Group III). In this group, we found that ARID3B, BCLAF1, CXorf56, DDX42, IBTK, ISY1, LIMA1, PGRMC1, PGRMC2, POLDIP3,

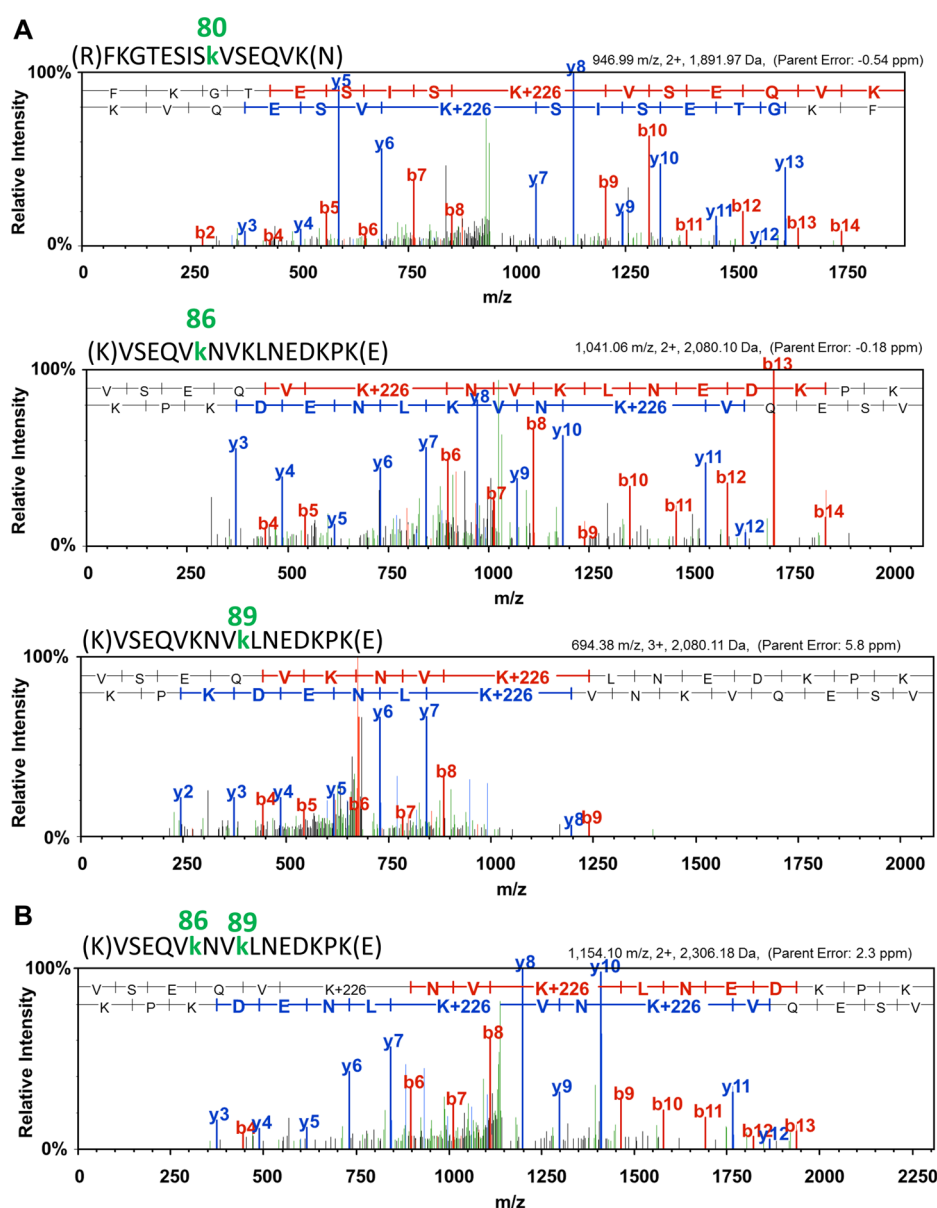


Figure 3. Representative MS/MS spectrum of labeled peptide of FKBP25. (A) MS/MS spectra of biotin-labeled peptides of K80-, K86-, and K89-labeled sites of FKBP25. (B) MS/MS spectrum of double biotin-labeled peptides (K86 and K89) of FKBP25. This spectrum was observed in the MS analysis of the FRB–pBirA transfected sample in the presence of rapamycin.

RPA1, RPAP3, SART1, SF3A1, SF3B2, SKP1, TFIP11, and USP15 were exclusively labeled by FRB–pBirA only in the absence of rapamycin. We hypothesize that these proteins should have a temporal interaction with FRB and could be dissociated by other rapamycin-induced interacting proteins in the presence of rapamycin.

From bioinformatics analysis, we found that the proteins in Group II and Group III have well-separated genetic networks; Group II genes have a functional linkage to the poly adenylation complex of mRNA, whereas Group III genes are connected to the mRNA splicing complex (see [Supporting Information](#)). From this analysis, we speculate that the rapamycin-controlled pathway might be related to different stages of mRNA processing.

FKBP25 Is the 25-kDa Protein Biotinylated by FRB–pBirA in the Presence of Rapamycin. Among the Group II proteins, FKBP25 is a 25-kDa protein that has a rapamycin-binding domain similar to other FKBP proteins. It shares an

FKBP-type peptidyl-prolyl isomerase (PPIase) domain at the C-terminus domain with other FKBP proteins such as FKBP12¹⁸ (Figure 4A). However, there has been no study investigating whether FKBP25 has a rapamycin-induced physical interaction with FRB in living mammalian cells reported to date. Thus, we designed several follow-up experiments to explore and confirm whether FKBP25 has a true rapamycin-induced interaction with FRB.

First, we performed an immunoprecipitation experiment to confirm that FKBP25 was enriched in the biotinylated protein pools of the FRB-R sample. For this study, we respectively enriched the biotinylated proteins of the FRB-R and FRB-NoR samples using streptavidin magnetic beads, and the enriched proteins were transferred to a nitrocellulose membrane and immunoblotted with anti-FKBP25. As shown in Figure 4B, FKBP25 was enriched in the SA-enriched sample of rapamycin-treated FRB–pBirA but not in the no-rapamycin treatment.

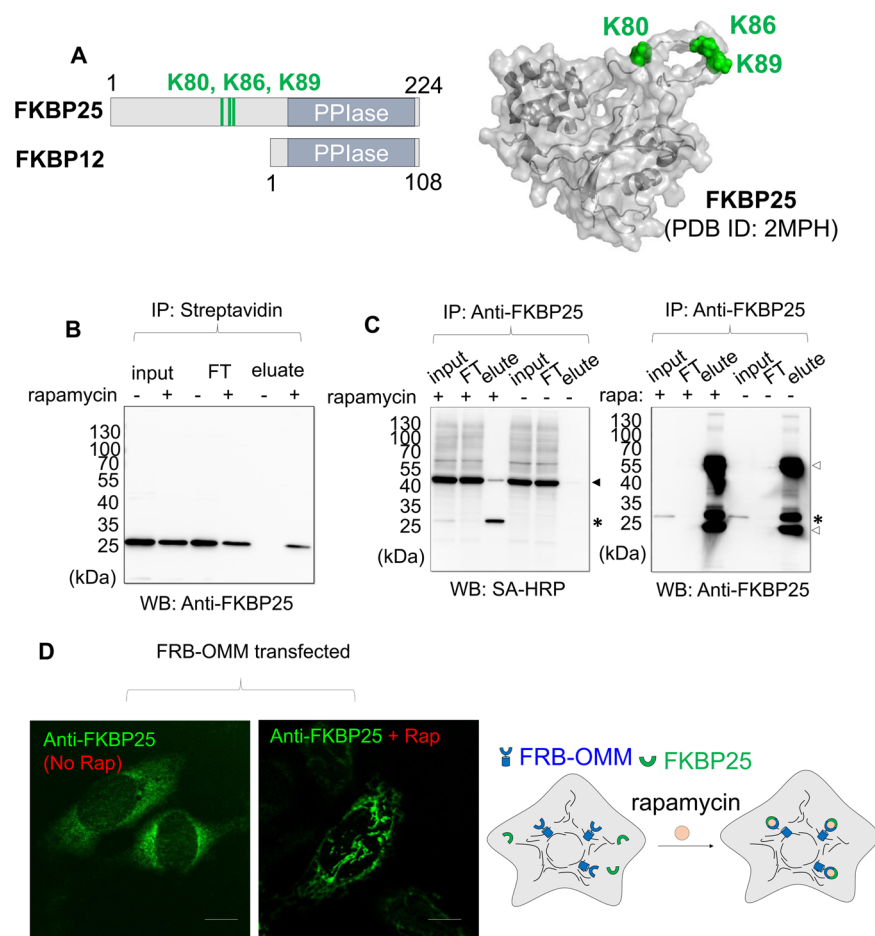


Figure 4. FKBP25 is a newly identified rapamycin-induced FRB-interacting protein. (A) MS-identified sites (K80, K86, K89) of FKBP25 biotinylated by FRB–pBirA. In the crystal structure of FKBP25 (PDB ID: 2MPH), the labeled lysine residues are green-colored. (B) Immunoprecipitation of biotinylated FKBP25 in the FRB-R sample by streptavidin. Streptavidin beads were used for pull-down, and anti-FKBP25 antibody was used for immunoblot. (C) Immunoprecipitation of biotinylated FKBP25 in FRB-R samples by anti-FKBP25. Anti-FKBP25 was used for pull-down, and streptavidin-HRP (SA-HRP) was used for immunoblot. Biotinylated FKBP25 is marked with an asterisk. Self-labeled FRB–pBirA is marked with a black arrow. The eluted heavy chain and light chain of the antibody are marked by blank arrows. (D) Confocal imaging of *in vivo* protein translocation of endogenous FKBP25 using transfected FRB-OMM (outer mitochondrial membrane) in HeLa cells. Scale bar = 10 μ m (left). Schematic representation is shown on the right.

We performed another immunoprecipitation experiment by using anti-FKBP25 to pull-down endogenous FKBP25 proteins from the whole cell lysate and checked whether endogenous FKBP25 was biotinylated using an SA-HRP Western blot. As shown in Figure 4C, endogenous FKBP25 in FRB–pBirA was biotinylated in the FRB-R sample, and endogenous FKBP25 in the FRB-NoR showed no biotinylation. In this experiment, we also found that a strongly biotinylated 25-kDa protein band in the whole cell lysate of the FRB-R sample was depleted in the flow-through sample with anti-FKBP25 immunoprecipitation, and enriched FKBP25 showed a strong biotinylated signal with the same molecular weight. This result provides further support that FKBP25 is the 25-kDa protein that was observed to be the most strongly biotinylated by FRB–pBirA in the presence of rapamycin (Figure 1C,D).

FKBP25 Physically Interacts with FRB in the Presence of Rapamycin in Living Cells. Next, we attempted to check whether FKBP25 physically interacts with FRB in the presence of rapamycin in a living cell. Previously, it has been demonstrated that FKBP12 and FRB physically interact in rapamycin-treated cells by imaging FKBP12-tagged protein translocation to artificially FRB-targeted spaces.^{22–25} Thus, we

employed this translocation assay to check for a protein–protein interaction between FKBP25 and FRB in living cells. For this assay, we employed FRB-OMM, which is a fusion construct of FRB to the OMM domain of monoamine oxidase that targets the outer membrane of the mitochondria.²² After transient expression of FRB-OMM, we checked the localization patterns of endogenous FKBP25 by incubation with an anti-FKBP25 antibody in rapamycin-treated and nontreated samples. As shown in Figure 4D, FKBP25 in the rapamycin-treated sample clearly localized in the mitochondria, but the same protein showed cytosolic localization without rapamycin treatment. This result indicates that FKBP25 and FRB physically interact only in the presence of rapamycin, as expected (Figure 4D). We further confirmed that the exogenously expressed FKBP25-tagged protein (FKBP25-V5-APEX2) is also translocated to FRB-OMM in the presence of rapamycin (Figure S1). This result indicates that FKBP25–FRB can be utilized as a genetically encoded chemical-induced protein dimerization tool, similar to FKBP12–FRB.⁴⁰

It is noteworthy that the immunofluorescence images of FKBP25 showed a clear cytoplasmic pattern and did not localize to the nucleus. Several earlier studies of FKBP25

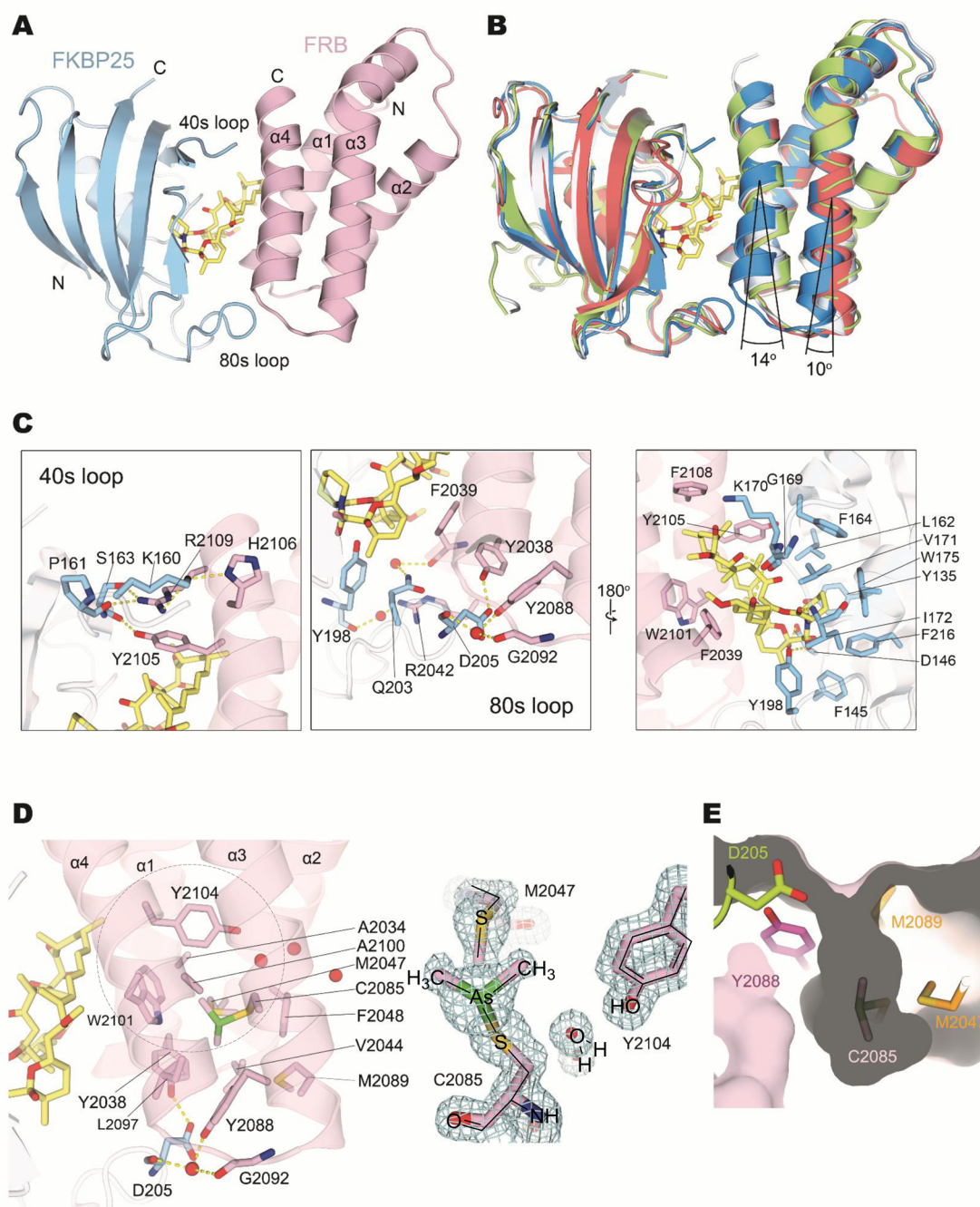


Figure 5. Crystal structure of FKBP25–FRB–rapamycin ternary complex. (A) Ribbon diagram shows the overall structure of the FKBP25 (blue)–FRB (pink)–rapamycin (yellow) ternary complex. The crystal structure of FKBP25–FRB–rapamycin was determined by molecular replacement using the FKBP12–FRB–rapamycin structure as a search model and refined with native data to 1.67-Å resolution. Rapamycin is shown as a stick model with oxygen and nitrogen atoms colored in red and blue, respectively. (B) Structural alignment among FKBP25–FRB–rapamycin (blue), FKBP12–FRB–rapamycin (red), FKBP51–FRB–rapamycin (green), and FKBP52–FRB–rapamycin (light blue) reveals that the overall structure of FKBP25 highly resembles other FKBP–FRB–rapamycin ternary complex structures. The molecular structure of rapamycin in FKBP12–FRB–rapamycin, FKBP51–FRB–rapamycin, and FKBP52–FRB–rapamycin is omitted in the figure for clarity. (C) Ribbon diagram shows the dimerization mediated by rapamycin (yellow) between FKBP25 (blue) and FRB (pink). Oxygen and nitrogen atoms are red- and blue-colored, respectively. The yellow dotted lines indicate intermolecular hydrogen bonds. (D) The picture shows C2085 covalently modified with an arsenic atom from cacodylic acid (see text for details). (E) Protein surface presentation shows hole formation in FRB by FKBP25–rapamycin complexation. C2085 is colored pink. Dimethyl arsenic group is colored blue. M2047 and M2089 are colored orange. Y2088 is colored magenta and D205 of FKBP25 is colored green.

proposed that this protein is a nuclear protein because it has been identified in the nuclear fraction of the cell lysate and possessed a predicted nuclear localization signal.^{41,42} However, immunofluorescence images of FKBP25 in our experiment

using HeLa cells and other reported immunofluorescence images of FKBP25 in other cell lines (e.g., U2OS, A431, U251MG) originating from different tissue samples support that FKBP25 is a cytoplasmic protein that is not localized in the

nucleus.⁴³ Thus, the physiological role of FKBP25 should be reconsidered as a rapamycin-induced FRB interactor in the context of the cytoplasm. Because FKBP25 has not been previously discussed as a rapamycin-dependent interactor for FRB in the cytoplasm, we performed further experiments to identify the crystal structure of the ternary molecular complex of FKBP25–rapamycin–FRB to reveal its physiological role in mTOR signaling.

Structural Basis for FKBP25 Binding to FRB Dependent on Rapamycin. To further investigate the binding of FKBP25 to FRB, we determined the crystal structure of the FKBP25–FRB complex in the presence of rapamycin at a 1.67 Å resolution (Figure 5A). The overall structure of FKBP25–FRB–rapamycin highly resembles the FKBP12–FRB–rapamycin, FKBP51–FRB–rapamycin, and FKBP52–FRB–rapamycin structures with root-mean-square deviations (RMSDs) of 1.0 Å, 1.2 Å, and 0.9 Å, respectively (Figure 5B).^{44,45} The crystal structures demonstrate that FKBP25 directly interacts with FRB in a rapamycin-dependent manner, such that the rapamycin is located in the center, capped by FRB and FKBP25 at each end. Because the binding conformation of the FKBP25–rapamycin–FRB complex is very similar to the FKBP12–rapamycin–FRB complex, we speculate that the physiological role of FKBP25 should be related to inhibit mTOR activity in the presence of rapamycin. Our hypothesis can be supported by previously reported *in vitro* experiment results which showed that FKBP25 inhibits mTOR activity with rapamycin ($IC_{50} = 2.61$ nM).⁴⁵

The direct interactions observed in the FRB–FKBPs (FKBP12/FKBP51/FKBP52) complex, mediated by the 40s and 80s loops of FKBP, as well as the rapamycin-dependent interactions are highly conserved in the FRB–FKBP25 complex (Figure 5A,C). However, unexpectedly the 80s loop of FKBP25 was found to be closer to the $\alpha 4$ helix of FRB compared to other FKBP when analyzed with structural alignment. For such proximal contact, novel interactions mediated by the D205 residue of FKBP25 are newly established. The D205 residue is absolutely conserved among FKBP25 orthologs in metazoan but is absent in other FKBP proteins, including FKBP12, FKBP51, and FKBP52 (Figure S2). In particular, the main chain of D205 from FKBP25 makes hydrogen bonds with the main chain of G2092 and the side chain of Y2088 from FRB through the buried water molecules. In addition, the side chain of D205 in FKBP25 makes hydrogen bonds with the side chain of Y2038 from FRB (Figure 5C). Interestingly, in order to make novel contacts, the axes of the $\alpha 3$ and $\alpha 4$ helices of FRB were pulled out around 10 deg toward the 80s loop of FKBP25 compared to the helices in other FKBP–FRB complexes (Figure 5B).

Surprisingly, the conformational changes of these helices in FRB generate a hole where C2085 of FRB is located at the bottom, and this residue was modified by dimethyl arsenic group (Figure 5D). Characterization of the arsenic atom in the protein complex was performed by anomalous scattering scanning and X-ray absorption spectroscopy (Figure S3). The dimethylarsenic group is known to be produced from the reaction between cacodylic acid and dithiothreitol in the crystallization buffer.⁴⁶ It was previously reported that dimethyl arsenic modification occurred with a reactive cysteine residue in the enzyme active site.⁴⁷ Furthermore, we found that this hole is a methionine-rich space given the presence of M2089 and M2047. Thioethers of these methionine residues may introduce transition metal ions from the surface to the bottom of the

hole.⁴⁸ Because this metalloid coordination at C2085 of FRB was not observed in ternary complex structures of other FKBP, we postulate that FKBP25 should play a unique physiological role related to metallomics in the mTOR signaling pathway.

To investigate the physiological roles of the predicted metal binding site of FRB in the complex with FKBP25, we performed site-directed mutagenesis (e.g., C2085A and M2047L) of the amino acid residues in the predicted metal binding sites of FRB. We measured the circular dichroism (CD) spectrum of purified C2085A-FRB, M2047L-FRB, and wild type FRB with FKBP25 depending on the metal treatment. As shown in Figure S6A, wavelength scanning of the complexes revealed that no secondary structures changed when the metal bound to the complex. We also measured the melting temperatures (T_m values) of the complexes in thermal denaturation experiments for checking structural stability of each complex. As shown in Figure S6B, the melting temperature of the FRB–rapamycin–FKBP25 ternary complex ($T_m = 71.06$ °C) was increased by approximately 5 and 17 °C compared to that of FRB ($T_m = 65.76$ °C) and FKBP25 ($T_m = 54.43$ °C) alone, respectively, suggesting that FKBP25 binding to FRB significantly increases the protein stability of the ternary complex (Figure S6B).

T_m values of the FKBP25–rapamycin–wild type FRB complex was slightly increased from 71.06 to 71.55 °C upon addition of the arsenic atom (Figure S6B). We also found that T_m value of C2085A-FRB–rapamycin–FKBP25 was increased from 70.68 to 71.03 °C upon addition of the arsenic atom, while M2047L-FRB–rapamycin–FKBP25 complex showed a decreased T_m value from 69.09 to 68.59 °C upon addition of the arsenic atom (Figure S6C,D). This result suggests that metal binding of the M2047 residue of FRB may contribute to the stability of the FRB–rapamycin–mFKBP25 ternary complex (Figure S6E).

The importance of metal coordination of FRB is further supported by the sequence conservation of M2047, which was predicted to be a key residue involved in metal coordination in FRB based on the crystal structure (Figure 5E). Surprisingly, M2047 is absolutely conserved among 10 other species, from metazoan to yeast (Figure S6F).⁴⁹ We also found M2047L-FRB and its ternary complex with rapamycin and FRB showed lower T_m values than those of wild type FRB and its ternary complex, which indicates this site mutation significantly affected the stability, although the secondary structure was unaffected by the mutation (Figure S6A,D,E). Collectively, these results suggest that the metal coordination with the M2047 residue play an important role to increase stability of the ternary complex of FRB–rapamycin–FKBP25. Further analysis is required to address the physiological functions of metal coordination to the mTOR complex in living cells.

DISCUSSION

In total, 18 different FKBP proteins are known to be expressed in various human tissues, and all of these proteins contain the PPIase domain.¹⁸ However, not all of these proteins show strong binding affinity toward rapamycin.⁵⁰ Among these proteins, FKBP25 and FKBP12 are known to be the best rapamycin binders (K_i of FKBP25 = 0.9 nM, K_i of FKBP12 = 0.26 nM).⁵¹ März et al.⁴⁴ reported that hFKBP12 and hFKBP25 showed similar half-maximal effective concentration (EC_{50}) values of rapamycin for ternary complex formation (3.8 nM for FKBP12, 4.3 nM for FKBP25), whereas other cytosolic FKBP showed low affinity (e.g., 25 nM for FKBP51 and 25.5

nM for FKBP52). We consider that the lower ternary protein complex binding affinity of FKBP51 and FKBP52 can explain why these proteins were not labeled by FRB–pBirA, because stronger competitor proteins (e.g., FKBP12 or FKBP25) were present in the cytoplasm.

Although FKBP12 was not identified in our data set, our results do not exclude the possibility that FKBP12 is a main interaction partner of FRB–pBirA in the presence of rapamycin, because FKBP12 is a short protein that lacks a flexible loop with an accessible lysine residue, which are also properties of FKBP25 (Figure 4A). This could be considered as a false-negative result of pBirA labeling, which requires an accessible lysine residue of proximal proteins. However, our data suggest that FKBP25 is likely to be one of the main interactors of FRB in the presence of rapamycin because FKBP25 has a strong possibility to be proximally biotinylated by FRB–pBirA even if FKBP12 is present as a strong competitor in the cytoplasm.

For comparison with other proximity labeling methods, we used FRB-conjugated engineered ascorbate peroxidase^{4–6} (FRB–APEX2) and determined whether FKBP25 was labeled in the presence of rapamycin. As shown in Figure S4 we found that FKBP25 was not biotinylated by FRB–APEX2. Because there are no or fewer accessible exposed tyrosine residues at the flexible loop of FKBP25, we postulated that proximity labeling by biotin-phenoxyl radical coupling should hardly occur.^{4–6} In contrast, there were several solvent-exposed lysine residues (K80, K86, and K89) at the loop, which would be favorable for proximity labeling by pBirA.

From a bioinformatics analysis of FKBP25, we determined that more than 10% of cancers originating from the pancreas, lung, and upper aerodigestive tract had significantly higher expression of FKBP25 (see Supporting Information). Thus, this high expression of FKBP25 in cancer tissues could make them a candidate for rapamycin or rapamycin analogue treatments for achieving efficient inhibition of mTOR activity, which is deeply related to tumorigenesis.¹⁵

CONCLUSION

We identified that FKBP25 is a strong interactor of mTOR protein in the presence of rapamycin in live cells through a new workflow designed to analyze labeled peptides from proximity labeling. We also successfully obtained the X-ray crystal structure of the ternary molecular complex of FKBP25–rapamycin–FRB. In this crystal structure, we could observe that metalloid modification on C2085 of FRB at the bottom of the hole which was induced by rapamycin–FKBP25. Our result implies that FKBP25 might play a unique role related to metallomics in the mTOR pathway.

EXPERIMENTAL SECTION

Proximity Labeling by FRB–pBirA. For the proximity labeling experiment in living cells, we prepared a pBirA fusion construct of the FRB domain at the C-terminus (FRB–pBirA) in a mammalian expression vector (pCDNA5) and evaluated the expression pattern and biotinylation activity of FRB–pBirA-transfected cells by immunofluorescence imaging experiments (Figure 1B). For this Western blotting experiment, FRB–pBirA was transiently expressed in HEK293T cells and incubated with 50 μ M biotin for 16 h at 37 °C. For the rapamycin-treated sample (FRB–R), the cells were treated with 100 nM of rapamycin for 16 h along with biotin, whereas in the no

rapamycin-treated sample (FRB–NoR), the cells were only treated with biotin at this step. After biotin labeling, the cells were sacrificed and the biotinylated proteins were analyzed by streptavidin-horseradish peroxidase (SA-HRP) Western blotting (Figure 1C,D). The detailed experimental procedures experiments are described in Supporting Information.

Mass Spectrometry Identification of Biotin-Labeled Sites by pBirA. The biotinylated peptides were eluted with a 95% formamide solution, which is acceptable for mass spectrometer injection. In the tandem mass spectrometry analysis of the SA-eluted peptides, a total of 4144 peptides containing a biotin-modified lysine (lysine+226 Da) residue were filtered from the total of four biological replicates of pBirA-labeled samples (FRB–R–Rep1, FRB–R–Rep2, FRB–NoR–Rep1, FRB–NoR–Rep2) since they showed the lowest 1.0% false discovery rate (FDR). We filtered these peptides with more than or equal to 2 exclusive spectral counts per labeled lysine site to obtain labeled peptidome information with higher confidence.

Circular Dichroism Analysis. Structural change and stability in the FRB, FKBP25, and FRB–rapamycin–FKBP25 ternary complex (15 μ M) were monitored by a circular dichroism (CD) spectrometer (Jasco J-815) at various wavelengths (190–260 nm). Conformational changes for each protein were also monitored at various temperatures by scanning with a CD spectrometer. The temperature was raised from 30 to 95 °C over a 13 min period with detection on 222 nm wavelength. All the samples used here were prepared in buffer A (25 mM sodium phosphate, 150 mM NaCl, pH 7.5). For incorporation of the arsenic atom to the FRB protein, we incubated purified proteins with 100 mM cacodylate pH 6.5 and 5 mM DTT for 2 h at 4 °C, and proteins were applied onto a HiTrap desalting column (GE Healthcare) equilibrated with buffer A. All mutants were generated by a PCR based site-directed mutagenesis method, and mutation in the FRB sequence was confirmed by DNA sequencing.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.6b00137.

All experimental details (PDF)

Table of mass characterization of labeled peptides (XLSX)

Table of supplementary results (XLSX)

Accession Codes

The coordinates and structure factors have been deposited in the Protein Data Bank (accession code 5GPG).

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

mTOR, mammalian target of rapamycin; pBirA, promiscuous biotin ligase; FRB, FK506-rapamycin binding; FKBP, FK506-binding protein; POI, protein of interest; SA-HRP, streptavidin-HRP

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