Rescue of Degradation-Prone Mutants of the FK506-Rapamycin Binding (FRB) Protein with Chemical Ligands


We recently reported that certain mutations in the FK506-rapamycin binding (FRB) domain disrupt its stability in vitro and in vivo (Stankunas et al. Mol. Cell, 2003, 12, 1615). To determine the precise residues that cause instability, we calculated the folding free energy (ΔG) of a collection of FRB mutants by measuring their intrinsic tryptophan fluorescence during reversible chaotropic denaturation. Our results implicate the T2098L point mutation as a key determinant of instability. Further, we found that some of the mutants in this collection were destabilised by up to 6 kcal mol\(^{-1}\) relative to the wild type. To investigate how these mutants behave in cells, we expressed firefly luciferase fused to FRB mutants in African green monkey kidney (COS) cell lines and mouse embryonic fibroblasts (MEFs). When unstable FRB mutants were used, we found that the protein levels and the luminescence intensities were low. However, addition of a chemical ligand for FRB, rapamycin, restored luciferase activity. Interestingly, we found a roughly linear relationship between the ΔG of the FRB mutants calculated in vitro and the relative chemical rescue in cells. Because rapamycin is capable of simultaneously binding both FRB and the chaperone, FK506-binding protein (FKBP), we next examined whether FKBP might contribute to the protection of FRB mutants. Using both in vitro experiments and a cell-based model, we found that FKBP stabilizes the mutants. These findings are consistent with recent models that suggest damage to intrinsic ΔG can be corrected by pharmacological chaperones. Further, these results provide a collection of conditionally stable fusion partners for use in controlling protein stability.

Introduction

Rapamycin is a potent immunosuppressant that binds tightly to the FK506-binding protein (FKBP).\(^1\) When bound to FKBP, rapamycin acquires affinity for a region of mammalian target of rapamycin (mTor) known as the FKBP-rapamycin binding (FRB) domain.\(^5\) Thus, rapamycin can bind two proteins at the same time and its coupling to the FRB domain is accompanied by recruitment of FKBP (Figure 1A). The most common form of FKBP is a 12kDa cis–trans prolyl isomerase that serves as a chaperone for newly synthesized polypeptides.\(^8\) Rapamycin binds within the active site of FKBP and inhibits isomerase activity. The other side of rapamycin binds in a cleft between two helices of FRB, which is an 89-amino acid, classical up–down four-helix bundle.\(^9\) In the ternary complex, rapamycin facilitates limited contacts between FRB and FKBP that further enforce tight binding.\(^9,10\) Because of these interesting binding characteristics, the FKBP and FRB proteins have been widely used as research tools.\(^11–21\) In these applications, rapamycin (or its synthetic derivatives) have been used to control the juxta-position of target proteins that are expressed as fusions to FKBP and FRB. This technology arose from versatile earlier methods that employ chemical inducers of dimerization (CIDs), such as dimeric versions of FK506, to bring together FKBP fusion proteins.\(^22\)

During recent attempts to generate an engineered version of the FRB domain, mutations at three positions within the rapamycin-binding interface (K2095P, T2098L, and W2101F)\(^23\) were found to supply selective affinity for a detoxified version of rapamycin, C20-methallylrapamycin (MaRap).\(^24–28\) For clarity, we apply the single-letter amino-acid code and refer to the wild-type protein as KTW and the mutant sequence as PLF. However, because PLF was identified in a screen, it was not clear if all three mutations are required for MaRap binding. To examine this, we generated individual point mutations and

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systematically explored how these changes affected binding to chemical partners. These studies identified the phenylalanine residue at residue 2101 as the key determinant of whether a FRB domain will accept MaRap.[27]

Interestingly, we found that the three mutations in PLF destabilize it relative to KTW. In vitro, PLF is more susceptible to thermal denaturation and, in cell lines and mouse fibroblasts, protein fusions to PLF are rapidly degraded compared to KTW protein fusions.[24] However, addition of rapamycin or MaRap to cells that express a fusion of PLF to the kinase, GSK3β, restores protein levels and kinase activity.[24] Upon removal of rapamycin or the addition of a competitor, stability is lost and the protein is degraded. Thus, rapamycin can be used to restrict the activity of a PLF-fusion protein to specific periods of time, a process that we term "chemically inducible stabilization". This method belongs to a growing family of chemically regulated degradation systems.[12,26–30]

Although inducible stabilization has applications in the study of protein function, it is unclear whether all three point mutations in PLF are required to damage stability because they were not initially selected for this purpose. Thus, we were interested in studying the origins of instability by assaying the effects of substitutions at various amino acid positions in FRB. During the course of this work, we learned that the T2098L mutation is principally responsible for the instability of PLF and we also made the surprising observation that some of the FRB mutants were destabilized by nearly 6 kcal mol\(^{-1}\) against KTW. To examine the impact of this instability in cells, we created a fusion between luciferase and the FRB mutants. In this study, addition of rapamycin recovered luciferase levels. Before a likely source for instability. Finally, we examined the contribution of FKBP to the recovery of unstable PLF mutants. In vitro and in cells, we found that recruitment of FKBP is strongly stabilizing.

Results and Discussion

Three mutations in FRB render it unstable compared to the wild type

To confirm the relative stabilities of KTW and PLF, we purified recombinant proteins from bacteria and measured their intrinsic tryptophan fluorescence during chaotropic denaturation (Figure 1B). The folded, wild-type FRB protein has been reported to be stabilized against the unfolded state by approximately 6–8 kcal mol\(^{-1}\) and our results were consistent with these observations. Moreover, comparing the ΔG of PLF and KTW revealed that the mutant was destabilized by 3.9 kcal mol\(^{-1}\). Thus, consistent with our previous findings, the three point mutations in PLF that were originally intended to provide specificity for synthetic rapamycin derivatives have the side effect of destabilizing the protein.

Design of mutations in the rapamycin-binding interface

Amino acids 2095, 2098, and 2101 of FRB are in the rapamycin-binding interface and the W2101F mutation is responsible for the binding selectivity of PLF for the rapamycin analogue, MaRap.[27] Therefore, we wondered whether this substitution also caused the destabilization of PLF. To address this, we generated the KTF mutant, which has the W2101F mutation but has the wild-type residues at positions 2095 and 2098. KTF was subjected to chemical denaturation and the ΔG value (and the ΔΔG relative to KTW) was determined (Figure 2B, i). Analysis of this data suggested that KTF was nearly as stable as KTW (ΔΔG = 0.7 kcal mol\(^{-1}\)); this indicates that the W2101F mutation does not give rise to the instability of PLF. Therefore, different amino acid substitutions must contribute to the instability and drug selectivity of PLF. Next, we generated a series of mutants in which amino acid 2095 was altered. The ΔG values for these mutants suggest that the instability of PLF is not engendered by the proline mutation (Figure 2B, i). For example, the PTF mutant was only mildly destabilized compared to KTW (ΔΔG = 0.5 kcal mol\(^{-1}\)). This result was surprising, as we initially considered the K2095P mutation, as a probable helix-breaker, to be the most dramatic of the three substitutions and therefore a likely source for instability. Finally, we examined the contribution of residue 2098. Interestingly, introduction of the T2098L point mutation alone (KLW) lowered the ΔG by 2.1 kcal mol\(^{-1}\) (or 55% of the total loss observed in PLF; Figure 2B, i). This result indicates that the T2098L substitution is the primary source of instability in PLF and that this residue has an important role in the folding of FRB.
Computational design of additional mutants

While our results suggest that residues within the rapamycin-binding region are important determinants of FRB stability, we also wanted to understand whether mutations in other regions of the protein would provide unstable derivatives. To explore this question, we selected additional residues to target using a computational approach. The protein design module of the ROSETTA molecular modeling program identifies amino acid sequences predicted to be most compatible with a given protein backbone. This program, therefore, permits the evaluation of the effects of amino acid mutations on protein stability, in silico. In this analysis, the protein backbone arrangement is held fixed, and the conformations of the altered (and neighboring) residues are determined by using rotamer-based Monte Carlo optimization. For the FRB domain, the effects of virtual mutagenesis were assessed for a large number of substitutions at a collection of amino acid positions. This analysis yielded tables of the intrinsic folding free energy predicted for each mutational outcome. From the resulting data tables, the top ~5% of mutations that predict substantial changes were selected (Figure 2B). We generated these mutations in the context of PLF to understand whether the alterations would synergize with those within the binding interface. Strikingly, these changes yielded proteins that vary in intrinsic folding energy over a range of approximately 6 kcal mol\(^{-1}\). Some of the mutations (e.g., I2111V) produced FRB domains that were similar to the wild-type in stability, while others (e.g., R2076A) were slightly less stable than PLF. Thus, this collection of ~20 mutants provided a spectrum of FRB proteins that varied in intrinsic folding energies.

Mutant FRB domains destabilize glutathione S transferase (GST)

We had previously determined that PLF could be appended to certain proteins and that the fusion becomes unstable. To explore whether this was a general property of unstable FRB mutants, we expressed our collection attached to the C terminus of glutathione S transferase (GST). We again used tryptophan fluorescence to monitor protein unfolding and, from this data, determined the \(\Delta G\) and \(\Delta \Delta G\) relative to a fusion of KTW to GST. Similar to what we observed for the free domains, the fusions varied in \(\Delta G\) and these results confirmed that the T2098L mutation is a key mediator of stability. Interestingly, the range of \(\Delta \Delta G\) observed was less than 5 kcal mol\(^{-1}\), compared to 6 kcal mol\(^{-1}\) for the free FRB domains (Figure 2B, i). This result suggests that GST might dampen the effects of tag instability and minimize the impact of mutations on the overall free energy. To further explore this relationship, we performed a crude comparison of the \(\Delta \Delta G\) of the free FRB domains and the corresponding FRB–GST fusions (Figure 3). A roughly linear relationship was evident, which suggests that the relative sta-

Figure 2. A series of mutations in the FRB domain reveals the origins of PLF instability. A) Replacing each of the individual PLF mutations in FRB suggests that the T2098L mutation contributes the bulk of the observed instability. B) Collection of mutants and their observed properties. The mutants with changes in the rapamycin-binding domain are shown on top and those with perturbation in other regions are on the bottom. Results of experiments involving measurement of \(\Delta G\) by intrinsic tryptophan fluorescence during chemical denaturation, ii) luciferase activity of luciferase-FRB fusions in two different cell lines, and iii) activation of an FKBP-rapamycin-FRB transcriptional switch in COS cells are shown. A full description of the experimental details can be found in the Experimental Section. All errors are standard deviation from the mean.
Fusing PLF to luciferase creates a useful platform for studying relative stability in cells

While measuring the stability of purified proteins in vitro provided insight into the source of stability in FRB proteins, we sought to correlate these results with observations from a cell-based system. Cells provide the opportunity to study the effects of destabilizing mutations on the susceptibility of the protein to proteolytic degradation and quality control. Based on our observations that FRB instability transferred to GST fusion proteins, we hypothesized that a fusion to firefly luciferase would be useful for this purpose. In this scenario, enzymatic activity of an unstable fusion between the FRB domain and luciferase would be low in the absence of rapamycin but protein levels and luminescence would increase with addition of drug. To explore this concept, the PLF domain was attached at either the C or N terminus of firefly luciferase. COS1 cells were transfected with these constructs, and luciferase activity was measured. Consistent with the design, activity was low in the absence of drug but addition of 5 nM rapamycin resulted in a concomitant induction of signal and protein levels (Figure 4). In addition, we found that the fold induction was dependent on both the amount of vector that was introduced and the drug concentration. Interestingly, the C- and N-terminal fusions behaved differently; in the absence of rapamycin, the basal level of activity of the luciferase–PLF was less than that of the PLF–luciferase. Rapamycin increased the luciferase activity in both cases, but the fold change was ninefold for luciferase–PLF and less than fivefold for PLF–luciferase. This difference did not reflect differences in binding to rapamycin, because the EC50 was similar for both (0.97 and 1.2 nM; Figure 4B). These results suggest that the luciferase–PLF fusion might be

Figure 3. Relative stability of the free FRB domain is preserved in a GST fusion. The △ΔG of the free FRB domains is shown plotted against the ΔΔG of the fusion. In both cases, the ΔΔG is obtained by subtracting the ΔG of the mutant from that of the wild type, KTW. Results are the average of at least two independent experiments performed in triplicate each. Error is compounded standard deviation of the mean and the trendline is the best fit linear trend.

Figure 4. Luciferase fusions to PLF are unstable and can be rescued by rapamycin. A) Fusions to PLF are unstable. COS cells were transiently transfected with the indicated amount of vector and the luminescence of lysates measured (see the Experimental Section). Results are the average of two independent experiments performed in triplicate each, and error is standard deviation of the mean. The inset is an anti-FRB Western blot, which shows that the protein level of the luciferase–PLF fusion is enhanced by the addition of rapamycin. This antibody also reacts with endogenous mTor, which serves as the loading control. B) Luminescence is dependent on the concentration of rapamycin. The half-maximal activities mirror published reports for rapamycin efficacy, which suggests that neither fusion is impaired in binding ability. Results are the average of two independent experiments performed in triplicate each and error is standard deviation of the mean. C) The folding free energy calculated for each FRB mutant in vitro can roughly predict the relative stability in cells. Results are the average of two independent experiments performed in triplicate each. Error is compounded standard deviation of the mean. The inset is a similar analysis performed using the GST-fusion data.
less stable than the inverse arrangement. Although the origin of this difference is not yet clear, the luciferase–FRB fusion was chosen for further investigation.

The ΔG calculated in vitro predicts stability in cells

To determine the effects of FRB mutants on luciferase stability in cells, we generated fusions to a set of 20 mutants and screened these by using the luminescence assay. In these experiments, the luciferase activity of cells treated with 5 nM rapamycin was compared to untreated controls and the fold induction was measured. The constructs were tested in both COS1 cell lines and mouse embryonic fibroblasts (MEFs) to explore potential cell-to-cell variations. When linked to luciferase, these tags provided proteins with an array of sensitivities to rapamycin (see Figure 2B, ii). In general, FRB domains that were less stable in vitro produced luciferase fusions more responsive to the addition of rapamycin (Figure 4C). These results support a hypothesis in which changes to the primary sequence of FRB that alter the intrinsic folding energy of the domain cause concomitant changes in their stability in cells. Moreover, although the error was high, the ΔG calculated in vitro was somewhat predictive of the relative stability in cells.

Binding of rapamycin to FRB mutants is largely unaffected by the destabilizing mutations

One concern we had about the luciferase experimental platform is that the observed effects on luminescence could reflect differences in the affinity for rapamycin. Namely, the destabilizing mutations might disrupt binding to the chemical partner. To address this, we generated fusions of FRB mutants to the transcriptional activator domain, VP16. Addition of rapamycin to cells expressing both an FKBP–GaL4 fusion and the FRB–VP16 protein causes drug-dependent recruitment of the VP16 domain to DNA, transcriptional activation and expression of a secreted alkaline phosphatase (SeAP) reporter gene.[27] In this system, KTW has an EC50 for activation of SeAP production that resembles the known affinity of the macromolecular interaction (both are around 0.8 nM). Thus, as a convenient measure of relative potency, we followed SeAP levels as a function of rapamycin concentration to calculate the EC50 for a subset of FRB mutants (see Figure 2B, iii). Although there were notable exceptions (e.g., the EC50 for PTF and ATF were approximately threefold higher than for the wild type), these results suggest that a majority of the mutants bind rapamycin with similar potency. Thus, we conclude that the observed changes in luciferase activity are principally a result of damage to FRB stability and not differences in drug binding.

Recruitment of FKBP helps stabilize a FRB mutant

Rapamycin is a bifunctional molecule that can bind to both FRB and FKBP at the same time. Earlier work suggested that FKBP might be required for full recovery of PLF’s stability.[24] However, to explore this mechanism further, we used intrinsic tryptophan fluorescence to study the denaturation of FRB in the context of either rapamycin alone, FKBP alone or the combination. Measuring ΔG in vitro, we confirmed that addition of rapamycin to the isolated PLF domain improved stability (Figure 5A). Next, we added equal molar concentrations of FKBP to PLF in the absence of rapamycin. After subtracting the signal attributable to FKBP, we noted an apparent increase in the stability of the FRB domains. This result is consistent with previous findings that FK506-binding proteins assist in the folding of some proteins.[35] Because FKBP contributes to formation of the high affinity ternary complex, we wondered if its proximity would further stabilize mutants. To test this idea, we pre-incubated FKBP with rapamycin and PLF and then studied the stability of the system.

We found that the combination of rapamycin and FKBP was the most protective (Figure 5). This result supports a model in which formation of the ternary complex (FKBP–rapamycin–FRB) shields FRB domains from degradation and provides a profloding environment. Further, because rapamycin inhibits the prolyl isomerase activity of FKBP, these results also suggest that enzymatic function is not required for enhanced stability.

![Figure 5. FK506-binding protein protects unstable FRB mutants. A) Denaturation curves of PLF mutant protein in the presence of either FKBP alone, rapamycin alone or the combination. Results are representative of two independent experiments. B) In COS cells, disrupting FKBP binding with FK506 decreases PLF stability. Results are the average of two independent experiments performed in triplicate each. Error is standard deviation of the mean.](image-url)
Rather, it is likely that formation of the tight complex is responsible for the observed protection of mutants. To explore this idea in cells, we introduced the luciferase–PLF construct into COS cell lines and treated them with either rapamycin or a combination of rapamycin and FK506. FK506 binds with high affinity to FKBP, but it does not share rapamycin’s affinity for the FRB domain. Thus, this compound acts as a competitive inhibitor of the rapamycin–FKBP interaction. Increasing the relative dosage of FK506 greatly reduced luminescence (Figure 5B). This result suggests that disrupting association with FKBP diminishes the protective effects gained by rapamycin. Together, these biochemical and cell-based experiments suggest that the ternary complex is effective at insulating unstable FRB mutants.

Conclusions

Previous work has indicated that three point mutations in the FRB domain produce a mutant, PLF, that is unstable and prone to degradation in cell lines and mouse fibroblasts.[12,24] This property has been used to regulate a PLF-targeted allele of GSK-3β in mice, and use of this system has given important insights into the roles of GSK-3β in skeletal development.[15] Thus, we were interested in exploring the origins of FRB’s stability in an attempt to expand the utility of this system. From the relative ΔG values for various FRB mutants, we conclude that the T2098L mutation is the key residue involved in destabilizing PLF (Figure 2A).

We recently reported that the identity of residue 2101 determines the type of rapamycin analogues that are recognized by a FRB variant.[27] For example, we found that FRB domains with a phenylalanine in the 2101 position have affinity for MaRap but not other analogues, such as C16(S)-butylsulfamido-rapamycin. Conversely, we found that changes to residue 2098 had less impact on selectivity.[27] Combined with the results of this study, these findings suggest that the stability and selectivity of FRB mutants can be modulated independently of each other. Specifically, residue 2101 appears to be most important for selectivity, while amino acid 2098 is a key mediator of stability. Because FKBP and FRB domains are widely used in biochemical and cell biological applications,[12,13,18] this property might facilitate the rational design of FRB mutants with properties (i.e., specificity, stability) that are desirable for specific applications.

After isolating the key residue that participates in PLF instability, we became interested in whether point mutations outside the rapamycin-binding interface would also impact stability. To explore this issue, we used a computational approach to identify new mutants. Specifically, we used the design capabilities of the ROSETTA protein-modeling program to predict the effects of a number of proposed amino acid substitutions. This approach allowed us to focus experimental efforts on a small set of mutations most likely to disrupt the stability of FRB. Some of these mutations, such as R2076A, slightly decreased the stability of PLF. However, these results were often not dramatic and many of the mutations had only modest effects on stability. Based on this limited study, we propose that mutations within the rapamycin-contacting region are better suited for generating conditional stability. One possible explanation for this result is that residues in direct contact with the small molecule are best positioned to undergo dramatic changes in local environment during binding.

Rapamycin is a bifunctional molecule: it binds both FRB and FKBP. The affinity of rapamycin for FRB is enhanced by ~2000-fold when the drug is first bound to FKBP.[10] This enhanced affinity is likely mediated by favorable protein–protein interactions, as seen in the structure determined by crystallography.[9,18] This interesting mechanism of binding prompted us to explore the contribution of FKBP to stabilizing FRB domains. We found that the interaction with FKBP amplified the protective effects of rapamycin both in vitro and in cells (see Figure 5). These results suggest that formation of the ternary complex is required for full stabilization.

Numerous disorders, such as cystic fibrosis and Gaucher disease, have been linked to loss-of-function mutations that disrupt the folding and/or trafficking of the mutant protein.[37] One strategy to combat these pathogenically unfolded proteins is to generate “chemical chaperones” or “pharmacological chaperones” that bind to and stabilize the protein in its active conformation.[38–41] Chemical chaperones are typically weak inhibitors and they are believed to stabilize their targets by binding in the active site and providing folding energy. Chemical chaperones are gathering significant attention as potential therapeutic agents and recent successes have focused attention on understanding the mechanisms by which they protect damaged proteins.[42] Compelling evidence in the Gaucher disease model has suggested that the intrinsic folding energy of the disease-causing protein is a good indicator of its activity in cell-based systems and in patients.[43,44] In those studies, the relative stability of the target in vitro was found to correlate with defects in vivo. Further, addition of a chemical chaperone recovered both biochemical stability in vitro and function in cells. This conclusion could have some general applicability, because we found that degradation-prone FRB mutants behaved similarly. Specifically, we found that the ΔG in vitro correlated to stability in cells, and a small-molecule ligand could recover stability in both experimental settings. Thus, these studies support a model in which binding of a chemical chaperone helps template the binding site of unstable proteins and overcome damage to intrinsic ΔG caused by mutations.

Experimental Section

Cloning: Point mutations in the FRB sequence were introduced by Quikchange mutagenesis (Stratagene) using the plasmids pS-Luc-FRB or pBJS-FRB-VP16 as templates. Sequences encoding the FRB mutants were amplified by PCR from the pS-Luc-FRB vector and ligated as C-terminal fusions into the BamH1 and EcoR1 sites of pGEX2t (Amersham Pharmacia). Mutant identities were confirmed by sequencing. It should be noted that we employed an 89-amino acid version of the FRB domain, and it is unclear if our findings are applicable to the longer version (93 amino acids) used in related strategies.[10] All the constructs were generated in the same parent vector and, thus, contain the same promoter and are likely maintained at the same copy number. This approach was taken to minimize differences in apparent degradation rate due to relative...
expression levels instead of intrinsic stability. Previous observations in our laboratories have noted significant effects on relative degrada-
tion rate due to differences in expression.

Protein expression and tryptophan fluorescence: GST-FRB pro-
teins were purified from E. coli BL21 and dialyzed into Tris (10 mM, pH 7.2) with NaCl (150 mM). The pGEX2t vector contains one unique thrombin-recognition site between the GST and FRB do-
mains, which permits the cleavage of the GST by using the biotiny-
lated thrombin kit (Novagen). Protein concentrations were deter-
mind by Bradford assay with BSA as a standard. The mutant do-
mains were expressed in good yields in bacteria, although many
required immediate use and they degraded upon prolonged stor-
gage (JEG, unpublished observation). All proteins were adjusted to
1000 mM in the dialysis buffer before 50 μL aliquots were transfer-
ted to Costar black opaque 96-well plates. To initiate denaturation,
50 μL of 2× GuHCl was added, the solution mixed and incubated
15 min at room temperature. Consistent with previous reports,[9]
denaturation of FRB domains was reversible under these condi-
tions. Tryptophan fluorescence (280 nm/330 nm) was measured in
a SpectraMax M2 (Molecular Devices) at 23°C. Rapamycin does not
interfere with fluorescence in this region. Fluorescence values at
eleven GuHCl concentrations in triplicate were used to calculate
ΔG values in PRISM 3 (GraphPad Software) according to previously
described equations.[10,11] In experiments with FKBP and/or ramap-
ycin present (see Figure 5), FKBP and/or PLF were present at 1 μM
and the final concentration of rapamycin was 10 μM. In parallel
control experiments, the signal corresponding to denaturation of
FKBP was monitored in the absence of FRB protein and this value
was subtracted from the experiments in which both proteins were
included. Rapamycin was added from a 10 mM stock in ethanol and
equivalent levels (0.1%) of solvent were applied to the control
samples.

Molecular modeling: The ROSETTA molecular modeling program
was used essentially as described.[46,47] The crystal structure 2FAP
was used as a template for all the design calculations. The effects
of mutations on the free energy of folding were assessed after op-
timization of side chain conformations with a Monte Carlo rotamer
placement algorithm.

Luciferase stabilization assay: COS1 cells were cultured in Dulbec-
co’s Modified Eagle Medium + 10% fetal calf serum and penicil-
lin/streptomycin at 37°C and 5% CO2. For transfection experi-
ments, DNA was resuspended in 20 volumes of OptiMEM and
vortexed briefly prior to addition of three volumes of FUGENE 6
(Roche). After a 15 min incubation, the mixture was applied drop-
wise to 10 cm plates containing approximately 80% confluent
COS1 cells. The following day, cells were plated into the wells of
24-well culture plates in 500 μL of culture media. Rapamycin was
added after cells regained adherence. 24 h later, cell lysates were
prepared and luciferase assays performed by using the Dual Lu-
ciferase Kit (Promega, Madison, WI). Experiments were repeated
at least three times in either duplicate or triplicate wells. The fold in-
duction values were normalized to an internal standard consisting
of Luciferase-PLF-transfected cells treated with rapamycin (5 μM).
Similar conditions were used in experiments that utilized MEFs.[12]

Transcriptional switch SeAP assays: SeAP assays with FRB mu-
tants fused to VP16 were performed as described.[27] Briefly, 1 x 10⁶
COS cells were transiently transfected by electroporation with 2 μg
of each of three components of a rapamycin-sensitive transcription-
switch: pBJS-Gal4-FKBP, pBJS-FRB-VP16, and a Gal4-SeAP re-
porter. Cells were placed in 96-well tissue culture plates and incu-
bated for 24 h prior to addition of rapamycin (serial twofold dilu-
tions, eight concentrations). After an additional 24 h, endogenous
phosphatases were denatured by treatment at 65°C for 2 h. Addi-
tion of methylumbiferylphosphate (50 μL, 1 mM; Sigma, St.
Louis, MO) in diethanolamine (pH 10) initiated the SeAP assay.
After 16 h at 37°C, fluorescence was measured at 355/460 nm in a
SpectraMax M2 multimode plate reader (Molecular Devices, Sunny-
vale, CA). From these curves, the rapamycin concentration required
for half-maximal SeAP activity was calculated in PRISM 3 (Graphpad
Software).

Abbreviations
FKBP: human FK506-binding protein 12, FRB: FK506-rapamycin
binding domain, MEM: modified Eagle medium, GST: glutathione S
transferase, Luc: firefly luciferase, GSK3β: glycogen synthase kinase
3β, mTor: mammalian target of rapamycin, Rap: rapamycin, GuHCl:
guainidinium hydrochloride, ApoE: apolipoprotein E, MEF: mouse
embryonic fibroblasts, ΔG: change in folding free energy, ΔΔG:
change in folding free energy relative to wild-type.

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