Mammalian target of rapamycin (mTOR) is a highly conserved eukaryotic protein kinase that coordinates cell growth and metabolism, and plays a critical role in cancer, immunity, and aging. It remains unclear how mTOR signaling in individual tissues contributes to whole-organism processes because mTOR inhibitors, like the natural product rapamycin, are administered systemically and target multiple tissues simultaneously. We developed a chemical-genetic system, termed selectOR, that restricts the activity of a rapamycin analog to specific cell populations through targeted expression of a mutant FKBP12 protein. This analog has reduced affinity for its obligate binding partner FKBP12, which reduces its ability to inhibit mTOR in wild-type cells and tissues. Expression of the mutant FKBP12, which contains an expanded binding pocket, rescues the activity of this rapamycin analog. Using this system, we show that selective mTOR inhibition can be achieved in Saccharomyces cerevisiae and human cells, and we validate the utility of our system in an intact metazoan model organism by identifying the tissues responsible for a rapamycin-induced developmental delay in Drosophila.

Results

Rapamycin's unusual mechanism of action presented an opportunity to combine chemical and genetic modifications to target mTOR in specific cell populations. Rapamycin alone...
has low affinity for mTOR (16). It is the rapamycin-FKBP12 complex that presents a complementary binding surface for the mTOR FRB domain to assemble into a ternary complex. Therefore, we predicted that modifying rapamycin such that it could no longer bind FKBP12 would render it ineffective at inhibiting mTOR and that restoring FKBP12-binding of this analog through mutation of the FKBP12 binding site would, in turn, rescue formation of the inhibitory ternary complex (Fig. 1A). Even when administered systemically, this rapamycin analog would inhibit mTOR only in cell populations engineered to express the mutant FKBP12.

We first set out to design rapamycin analogs with reduced affinity for FKBP12. Reactions have been reported for direct modification of rapamycin at C-16, C-20, C-32, and C-40 (17–20). Crystal structures of the rapamycin-FKBP12 complex show that the C-32 ketone is the only position located inside the binding pocket, where it is closely flanked by the side chains of F46 and V55 (21). Reaction of rapamycin with an O-functionalized hydroxylamine generated a separable mixture of the corresponding E- and Z-oxime at the C-32 position (Fig. 1B). We constructed a library of 38 (19 substituents × 2 isomers) rapamycin analogs, or Rapa* compounds, with varying size, geometry, and hydrophobicity to probe the stringency of the FKBP12 binding site (Fig. 1C).

We selected Saccharomyces cerevisiae as a model to test the rapamycin analogs because of its exquisite growth sensitivity to rapamycin and the high conservation of its mTOR and FKBP12 orthologs. Knockout of fpr1, the yeast ortholog of FKBP12, conferred resistance to rapamycin in a disk diffusion assay, and introduction of human FKBP12 rescued full sensitivity (22) (Fig. 2A). We also introduced a series of FKBP12 mutants with substitutions to smaller amino acids at F46 or V55 to create space in the binding pocket to accommodate the additional bulk of the rapamycin analogs. We expected that these mutations would be tolerated because it has been shown previously that mutations to F46 or V55 have only a modest effect on FKBP12 rotamase activity (23). Immunoblots against a C-terminal influenza hemagglutinin (HA)-tag showed that expression of FKBP12 mutant proteins was reduced compared with wild-type FKBP12 (Fig. 2B). This suggests that the mutant proteins are somewhat destabilized despite their reported enzymatic activity.

We determined the sensitivity of the different FKBP12-expressing yeast strains to the panel of rapamycin analogs by measuring the zone of growth inhibition caused by each compound in a disk diffusion assay (Fig. 2C). Nearly all rapamycin analogs failed to inhibit growth in the wild-type FKBP12 strain, indicating that even small oximes at C-32 are sufficient to disrupt FKBP12 binding and prevent mTOR inhibition. A subset of FKBP12 mutants rescued sensitivity to some of the rapamycin analogs. The combination of Rapa*-3Z and FKBP12 V55G displayed the highest level of inhibition, similar to that of the rapamycin and wild-type FKBP12 combination. Cyclic and large, branched analogs were poorly tolerated across the panel of mutants, while short linear substituents exhibited activity exclusively with the V55 mutants. In each case, the Z isomer outperformed its E counterpart, suggesting that the geometry of this isomer is more closely complemented by the pocket created by mutating V55. Interestingly, rapamycin retained activity, albeit reduced, in the mutant strains, which indicates that the mutations did not preclude rapamycin binding or ternary complex formation.

We next sought to quantify and further characterize the selectivity of the rapamycin analogs for mutant FKBP12.

![Fig. 1. Design and synthesis of an orthogonal rapamycin analog. (A) Rapamycin forms an inhibitory complex by binding FKBP12 and mTOR FRB domain. Rapa*, a “bumped” analog of rapamycin, does not bind wild-type FKBP12 but does bind the “hole” mutant FKBP*. (B) Synthetic scheme for oxime ligation of rapamycin to generate Rapa* Z- and E-isomers. (C) Assembled Rapa* library containing various oxime R-groups.](https://doi.org/10.1073/pnas.2204083119)
biochemically. Using time-resolved fluorescence resonance energy transfer (TR-FRET), we measured the drug-induced association of mTOR FRB with wild-type or mutant FKBP12 (Fig. 3A). Each of the rapamycin analogs that displayed high FKBP12 V55G selectivity in yeast also selectively induced the mutant ternary complex in vitro, whereas rapamycin was not selective toward the wild-type complex (Fig. 3B). We next determined whether Rapa*-3Z could be used to selectively inhibit mTOR in human cells expressing mutant FKBP12. Achieving selectivity in human cells is more challenging due to the fact that the human genome encodes 14 different FKBP proteins (24). Most of these family members are known to bind rapamycin (25), and several may contribute to rapamycin-dependent mTOR inhibition (26). Using the Flp-In T-REx system, we generated a 293T cell line with inducible overexpression of HA-tagged FKBP12 V55G. We treated this mutant cell line and the parental cell line with Rapa*-3Z for

![Fig. 2.](https://www.pnas.org)
24 h and measured mTOR pathway outputs by Western blot (Fig. 4A). Rapa*-3Z inhibited mTOR signaling selectively in the mutant cells over a wide dose range. Phospho-S6K and phospho-S6, both downstream of mTORC1, were inhibited the mutant cells over a wide dose range. Phospho-S6K and phospho-S6, both downstream of mTORC1, were inhibited with EC50 of 1.0 nM and 1.3 nM, respectively, in the mutant cells compared with 76 nM and 121 nM in the wild-type cells (Fig. 4A and SI Appendix, Fig. S3). This shows nearly a 100-fold increase in sensitivity in the mutant-expressing cells. Phosphorylation of the mTORC2 substrate, Akt, was less sensitive overall to inhibition, a phenomenon that has also been observed with rapamycin (27, 28), but Rapa*-3Z selectively reduced Akt phosphorylation in the mutant 293 cells at higher doses (Fig. 4A).

Interestingly, we observed ligand-dependent stabilization of FKBP12 V55G (Fig. 4A). A C-terminal HA-tag allowed us to detect expression of the mutant protein and also decreased the protein’s electrophoretic mobility enough to resolve it from wild-type FKBP12. Mutant expression was well below that of wild-type FKBP12 in the dimethyl sulfoxide (DMSO)-treated cells, but in Rapa*-3Z-treated cells, expression increased dramatically. This also supports the conclusion that the V55G mutation is somewhat destabilizing to FKBP12. However, even low levels of FKBP12 V55G appear to be sufficient to sensitize cells to inhibition by Rapa*-3Z.

Due to mTOR’s central role in cell growth, its inhibition causes a decrease in cell proliferation. We observed that Rapa*-3Z selectively inhibited growth of mutant-expressing 293 cells after 72 h treatment, while rapamycin inhibited mutant and wild-type cells equally (Fig. 4B). This result was replicated in the breast cancer cell line MCF7, where Rapa*-3Z activity was restricted to the mutant FKBP12-expressing cells (Fig. 4C). To confirm that the observed growth inhibition was mediated through mTOR, we also tested Rapa*-3Z on MCF7 cells that harbored an mTOR mutation in the FRB domain, F2108L, that confers resistance to rapamycin-based inhibitors (29, 30). Rapa*-3Z had no effect on the growth of these cells (Fig. 4C).

One advantageous feature of small molecules is their ability to rapidly inhibit a protein target. This is especially important for signaling kinases, like mTOR, which are often regulated on the order of minutes rather than hours or days. After treatment with 10 nM Rapa*-3Z, mTOR signaling was rapidly inhibited in mutant FKBP12 293 cells. The time to half-maximal inhibition of S6 phosphorylation was 31 min (Fig. 4D and SI Appendix, Fig. S4). At this dose, the wild-type 293 cells were unaffected, demonstrating that mutant FKBP12 expression enables specific and rapid inhibition of the mTOR pathway by Rapa*-3Z.

To evaluate the utility of this selecTOR system in an intact model organism, we placed the human FKBP12 V55G coding sequence downstream from a GAL4-responsive upstream activating sequence (UAS) and used this construct to create transgenic flies (Fig. 5A). We then examined the influence of Rapa*-3Z on a developmental defect caused by TOR pathway inhibition. Previous work has shown that rapamycin treatment dramatically delays or abolishes larval development through inhibition of TOR pathway activity (31–33). As expected, we also observed profound inhibition of larval development upon rapamycin treatment of larvae (Fig. 5 B and C). However, we detected no effect of Rapa*-3Z on larval development in wild-type animals, even at high concentrations (SI Appendix, Fig. S5).

Ubiquitous expression (da-GAL4) of the FKBP12 V55G protein also had no apparent effect on larval development in the absence of Rapa*-3Z (Fig. 5 B and C, and SI Appendix, Fig. S6). However, Rapa*-3Z treatment of transgenic animals ubiquitously expressing the FKBP12 V55G protein potently inhibited larval development.
To directly compare the effective dose of Rapa*-3Z on larval development with that of rapamycin, we performed a dose-response experiment (Fig. 5 D–F). This work indicated that the dose of Rapa*-3Z necessary for complete inhibition of larval development in FKBP12 V55G animals was 100 μM, while 1 μM of rapamycin was required to achieve complete inhibition in wild-type flies. This difference in potency is likely due to the decreased relative permeability and bioavailability of Rapa*-3Z as observed by parallel artificial membrane permeability assay and Caco-2 permeability measurements (SI Appendix, Fig. S7). Further experiments indicate that Rapa*-3Z is fully stable under the conditions of our experiments for at least 15 d (SI Appendix, Fig. S8). Moreover, treating wild-type larvae with Rapa*-3Z after prolonged storage of the compound under experimental conditions had no apparent effect on larval development, indicating that Rapa*-3Z does not decompose to rapamycin under these conditions (SI Appendix, Fig. S8).

Previous work on the influence of rapamycin on larval development suggested that the cause of the larval developmental delay could originate in the musculature, nervous system, and/or ring gland, where the larval molting hormone ecdysone is produced (31–33). To address this matter, we expressed FKBP12 V55G using GAL4 drivers specific for one of these three tissues and then treated the animals with Rapa*-3Z. Specifically, we used the muscle-specific mhc-GAL4 driver, pan-neuronal nSyb-GAL4 driver, and ring-gland-specific plm-GAL4 driver (Fig. 6A). We treated these animals with 100 μM Rapa*-3Z, the minimal dose that completely prevented pupal formation 9 d after egg laying when FKBP12 V55G was expressed ubiquitously. We found that animals expressing FKBP12 V55G in the ring gland failed to pupate 9 d after egg laying in the presence of Rapa*-3Z (Fig. 6B and O). We detected similar, but less severe, inhibition of pupal formation in Rapa*-3Z-treated animals expressing FKBP12 V55G in the musculature and nervous system. To verify these findings, we repeated this work using an independent collection of GAL4 drivers specific for the ring gland (2–286-GAL4), musculature (Mef2-GAL4), and nervous system (elav-GAL4). Consistent with our previous results, we found that expression of FKBP12 V55G in the ring gland nearly eliminated the larval-to-pupal transition in the presence of Rapa*-3Z, with significant, but lesser, inhibition conferred by expression of FKBP12 V55G in the musculature and nervous system (SI Appendix, Fig. S9). Thus, our findings suggest that rapamycin’s effects on Drosophila larval development are mediated primarily by TOR pathway inhibition in the ring gland, but also in the musculature and nervous system.

**Discussion**

Rapamycin remains a promising therapeutic across broad indications, including cancer and aging, but in many instances, the tissues responsible for the beneficial effects of rapamycin are unknown, and unwanted side effects in off-target tissues, such as immunosuppression and metabolic dysregulation, limit the agent’s therapeutic potential. Experimental methods to investigate these questions are limited because genetic perturbation of mTOR does not recapitulate pharmacological inhibition. Therefore, here we describe selectTOR, a novel chemical-genetic strategy to target the activity of a rapamycin analog to specific cell populations. We determined that the rapamycin derivative Rapa*-3Z has greatly reduced ability to bind wild-type FKBP12 and inhibit mTOR in *S. cerevisiae*, mammalian cells, and *Drosophila*. We identified a single mutation in FKBP12 that restores Rapa*-3Z binding and rescues inhibition of mTOR. We showed that expression of mutant FKBP12 selectively sensitizes cells and tissues to inhibition by Rapa*-3Z. Finally, we used selectTOR to identify tissues that are responsible for the effects of TOR pathway inhibition on *Drosophila* larval development.
Previous bump-hole orthogonal rapamycin analogs focused on reengineering the rapamycin-FRB binding interface to prevent inhibition of native mTOR (34, 35). Adapting this system for tissue-specific targeting of mTOR would require tissue-specific knock-in or overexpression of mutant mTOR, potentially altering mTOR function in an unexpected manner. In contrast, our approach does not alter mTOR, which allows it to function as the wild-type kinase until the moment of inhibitor treatment. Additionally, mutant FKBP12 operates though a gain-of-function mechanism, so it can be simply overexpressed to sensitize the cells to inhibition, bypassing the need to install a mutation at the native FKBP12 locus.

By leveraging the specificity and modularity of genetics with the temporal control and therapeutic relevance of pharmacology, we hope to have built a tool that can reveal improved rapamycin-based therapeutic strategies and create a clearer picture of mTOR function in an unexpected manner. In contrast, our approach does not alter mTOR, which allows it to function as the wild-type kinase until the moment of inhibitor treatment. Additionally, mutant FKBP12 operates though a gain-of-function mechanism, so it can be simply overexpressed to sensitize the cells to inhibition, bypassing the need to install a mutation at the native FKBP12 locus.

Once the relevant tissue targets have been identified, one could imagine applying a number of approaches to target specific tissues pharmacologically. Physicochemical properties can be tuned to modulate drug partitioning through natural barriers such as the blood-brain-barrier, as seen with second-generation antihistamines (39, 40). Drug conjugation can take advantage of endogenous receptors to achieve preferential uptake in specific tissues (41, 42). Additionally, the use of nanoparticles or other delivery methods can promote both passive and active uptake of a drug into a target tissue (43).

The chemical-genetic approach applied here to rapamycin could be generalized to other “molecular glue” small molecules that rely on auxiliary proteins. The natural products FK506 and cyclosporin A depend on FKBP12 and cyclophilin, respectively, to bind and inhibit calcineurin (44), and PROTACs require binding to an E3 ligase to degrade their target protein (45). Making these molecules dependent on a mutant auxiliary protein would allow their activity to be targeted to specific cell populations, which may reveal new therapeutic modalities or uncover tissue-specific functions of their respective protein targets.

Materials and Methods

Chemical Synthesis. All Rapa* compounds were prepared by similar procedure with respective hydroxyamine hydrochloride derivatives. Rapa*—3Z was prepared as follows. Rapamycin (50 mg, 0.055 mmol) and ethoxyamine hydrochloride (8 mg, 0.082 mmol) were dissolved in methanol (2 mL). Pyridine (6.6 µL, 0.082 mmol) was added by pipette, and the reaction was stirred at room temperature for 24 h.

**Fig. 5.** Rapa*-3Z inhibits larval development in transgenic animals expressing mutant FKBP12 V55G. (A) Schematic diagram of the GAL4-responsive FKBP12 V55G transgene. (B) Vials illustrating the number of control (w1118) and transgenic animals (FKBP12 V55G) that develop to the pupal stage in the presence and absence of 10 µM rapamycin or 100 µM Rapa*-3Z. Images were captured 9 d after egg laying. (C) The relative number of pupae detected 9 d after egg laying in animals of the indicated genotypes in the presence and absence of 10 µM rapamycin or 100 µM Rapa*-3Z. (D–F) Dose-response effect of Rapamycin (D) and Rapa*-3Z (E and F) on the development of control (w1118; D and E) and transgenic (FKBP12/V55G; F) larvae 9 d after egg laying. At least three independent experiments were performed (n = 3). Significance was determined by one-way ANOVA multiple comparison test, ns = not significant; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001.
The crude material was purified by preparative reverse-phase high-performance liquid chromatography (50–100% CH3CN in water containing 0.1% formic acid, RediSep C’18 prep column). Z-repressor product eluted before E-repressor. Desired fractions were combined and lyophilized to give the desired product as a white powder (36 mg, 69% yield). Liquid chromatography-mass spectrometry (ESI+; [M+Na]+) m/z = 979.6, 1H NMR (400 MHz, chloroform-d, SI Appendix, Fig. S10).

Yeast Stocks and Maintenance. BY4741 fpr1::URA3 yeast were generated by homologous recombination, selected on Ura- synthetic complete medium and subsequently grown in yeast extract-peptone-dextrose (YPD) medium (46). Human FKBP12 coding sequence was cloned via Gibson assembly into a pCH043 yeast expression vector, which had been previously modified with an HIS3 selection open reading frame (ORF). The C-terminal HA-tag and F46/V55 mutations were introduced by site-directed mutagenesis PCR. Plasmids were transformed into the BY4741 fpr1::URA3 strain and selected on His- synthetic complete medium. Isolated strains were verified by plasmid sequencing. To prepare yeast protein lysates, 2 optical density (OD) equivalents from an overnight culture was resuspended in 200 μl 0.1 M NaOH and incubated at room temperature for 15 min. Cells were pelletted, resuspended in 50 μl 2x SDS loading dye, and heated at 95 °C for 5 min. Following centrifugation at 20,000 × g for 10 min, supernatant was saved for analysis.

Disk Diffusion Assay. Yeast strains containing FKBP12-expression vectors were shaken overnight at 30 °C in 3 mL His- synthetic complete media. 0.1 OD of culture was spun down, resuspended in 1 mL sterile water, and 100 μl of spread was on His-synthetic media containing galactose/raf- and Anti-HA-XL665 (Cisbio) were diluted to 0.625 μg/mL and 5 μg/mL, respectively, in assay buffer. Rapa* compounds and FK506 were diluted to 50 μM and rapamycin to 5 μM in assay buffer containing 5% DMSO, and threefold serial dilution series were made from these solutions. We mixed 4 μl diluted FKBP12-HA protein, 4 μl diluted GST-mTOR, and 4 μl of compound or DMSO control in a well of a black low-volume 384-well plate (Corning 4514), and the mixture was incubated at room temperature for 1 h. We then added 4 μl of Anti-GST-Tb, and 4 μl Anti-HA-XL665, and the mixture was incubated at room temperature for an additional 1 h. Time-resolved fluorescence was read on a Tecan Spark 20M plate reader with the following parameters: Lag time: 60 μs; Integration time: 500 μs; Read A: Excitation filter 320 (25) nm, Emission filter 610 (25) nm, Gain 130; Read B: Excitation filter 320 (25) nm, Emission filter 665 (8) nm, Gain 165. Three replicates were performed for each assay condition.

X-ray Crystallography. 2 μL of 100 mM Rapa* 3Z in DMSO was added to 100 μl of 1 mM FKBP12 V55G in 20 mM Tris pH 8.0 and incubated overnight at 4 °C. Precipitate was removed by centrifugation at 20,000 × g for 10 min at 4 °C. In a 15-well hanging drop plate with 500 μl well solution (0.1 M sodium tartrate, 18% PEG 3350), drops were set with 1 μl protein solution and 1 μl well solution and incubated at room temperature for 4 d. 10 μL cryo buffer (0.1 M sodium tartrate, 18% PEG 3350, 25% glycerol) was added to drops before crystals were looped and flash frozen in liquid nitrogen. Data were collected at Beamline 8.2.2 of the Advanced Light Source (Lawrence Berkely National Laboratory). Data were indexed and integrated using XDS (47). Molecular replacement was performed using Protein Data Bank 1FKB as a search model, and the model was refined and built using PHENIX (48) and Coot (49). Structures were visualized with ChimeraX (50).

Cell Culture. All cell lines were maintained in DMEM (Gibco) supplemented with 10% heat-inactivated FBS (Axenia Biologics) at 37 °C in 5% CO2. Flp-In T-Rex 293 cells were obtained from Thermo Fisher supplemented with 15 μg/mL blasticin and 100 μg/mL zeocin. Flp-In T-Rex 293 FKBP12 V55G cells were supplemented with 15 μg/mL blasticin and 150 μg/mL hygromycin B. FKBP12 V55G expression was induced with 1 μg/mL doxycycline 24 h prior to drug treatments. MCF7 cells were obtained from ATCC (American Type Culture Collection), and MCF7 mTOR F2108L cells were described previously (30). When indicated, cells were treated with drugs at 60–80% confluency at a final DMSO concentration of 0.1%. At the end of the treatment period, cells were placed on ice and washed once with PBS. Cells were scraped with a spatula and lysed in

Fig. 6. TOR pathway inhibition in ring gland, musculature, and nervous system delayed larval development in flies. (A) Schematic representation of FKBP12 V55G expression, ubiquitous (da-GAL4), musculature (mhc-GAL4), ring gland (phm-GAL4), or nervous system (nSyb-GAL4). (B) Vials illustrating the number of pupae detected 9 d after egg laying in animals of the indicated genotypes treated with 100 μM Rapa* 3Z. (n = 3). Significance was determined by group comparison via two-way ANOVA. ****P ≤ 0.0001; ***P ≤ 0.0001.
Gel Electrophoresis and Western Blot. SDS-PAGE was run with Novex 4–12% Bis-Tris gel (Invitrogen) in MOPS running buffer at 200V for 40 min. Samples were loaded at 20 μg lystate per lane. Protein bands were transferred onto 0.2 μm nitrocellulose membranes (Bio-Rad) using wet tank transfer apparatus (Bio-Rad Criterion blotter) in 1x TOWBIN buffer with 10% methanol at 75V for 45 min. Membranes were blocked in 5% BSA-TBST (tris-buffered saline, 0.1% Tween 20) for 1 h at room temperature. Membranes were cut into separate strips for each primary antibody. Primary antibody binding was performed with the indicated antibodies at manufacturer’s recommended dilution in 5% BSA-TBST at 4 °C overnight. After three 5 min. washes with TBST, secondary antibodies (Li-COR) were added as solutions in 5% skim milk-TBST at 1:10,000 dilutions. Secondary antibodies were incubated for 1 h at room temperature. Following three 5 min. washes with TBST, membranes were imaged on a Li-COR Odyssey fluorescence imager. Image contrast was adjusted independently for each strip using the “Auto” adjustment function in Fiji. Blot quantification was performed in Fiji, and data were graphed and fit in Prism 9 (GraphPad). The following primary antibodies were obtained from Cell Signaling Technology (CST), Abcam (ab), and ProteinTech (PT): S6 (CST 2317), phospho-S6 S240/244 (CST 5364), S6K (CST 2708), phospho-S6K T389 (CST 9206), Akt (CST 2920), phospho-Akt S473 (CST 4547) and GAPDH (PT 60004). For detection of phosphorylated and total protein, primary antibodies for phospho-Akt Akt, phospho-S6K/S6K, or phospho-S6/S6 were combined and detected using a mixture of species-specific secondary antibodies with orthogonal fluorescence to allow simultaneous detection of phosphorylated and total protein from a single membrane, or membrane was stripped (Cell Signaling Technology) after detection of phosphorylated protein and then reblocked and reprobed with primary antibody for total protein.

Cell Viability. Cells were seeded into 96-well tissue-culture treated plates (Greiner) and were allowed to incubate for 24 h. Cells were treated with indicated compounds and incubated for 72 h (100 μL final well volume, 0.1% DMSO). Cell viability was assessed by CellTiter-Glo (Promega). Assay reagent was diluted 1:4 in PBS containing 1% Triton X-100, and 100 μL was added to each well and mixed on a plate shaker for 20 min. Luminescence was measured on a Tecan Spark 20M.

Fly Stocks and Maintenance. All Drosophila stocks and crosses were maintained on standard cornmeal-molasses food at 25 °C using a 12-h light/dark cycle. To generate UAS-FKB12 V55-HA transgenic flies, human FKBP12 V55-HA ORF was cloned into a pUAST-atb vector between EcoRI and XhoI restriction sites. This construct was then integrated into an atb8 site using phiC31 integrate with the assistance of Rainbow Transgenic Flies, Inc. The driver stocks daGAL4 (ubiquitous), elavGAL4 and nSybGAL4 (neuronal-specific), Me2GAL4 and mhcGAL4 (muscle), and 2-286-GAL4 and phmGAL4 (ring-gland-specific) were obtained from Bloomington Drosophila Stock Center. The crossing schemes used in this study are depicted in SI Appendix, Fig. S11. For rapamycin or Rapa*32 related experiments, fly food was prepared freshly 24 h before each experiment.

Larval Development Experiments. For larval development assays, UAS-FKB12 V55-HA transgenic flies were set up for crosses with driver flies in freshly prepared fly food containing either rapamycin or Rapa*32 (dissolved in ethanol). Fly food containing ethanol vehicle was used as control. All images were captured on day 9 after setting up the crosses unless otherwise mentioned, and pupae were counted on day 9. Three biological replicates were used for each experiment. Data were analyzed using Prism 9.0 (GraphPad).

Data, Materials, and Software Availability. All study data are included in the article and/or SI Appendix. X-ray crystallography data have been deposited in Protein Data Bank [PDB ID 7UBD (51)].

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