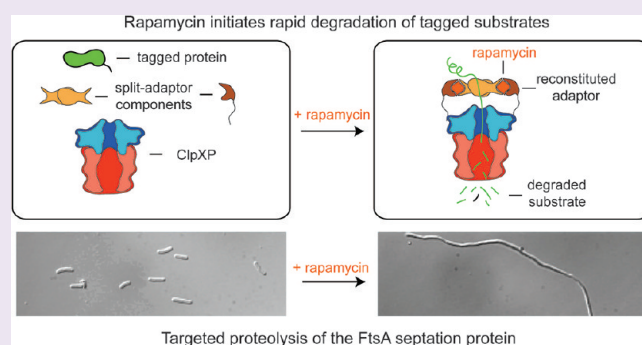


Small-Molecule Control of Protein Degradation Using Split Adaptors

Joseph H. Davis,^{†,§} Tania A. Baker,^{†,‡} and Robert T. Sauer^{*,†}[†]Department of Biology and [‡]Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States

ABSTRACT: Targeted intracellular degradation provides a method to study the biological function of proteins and has numerous applications in biotechnology. One promising approach uses adaptor proteins to target substrates with genetically encoded degradation tags for proteolysis. Here, we describe an engineered split-adaptor system, in which adaptor assembly and delivery of substrates to the ClpXP protease depends on a small molecule (rapamycin). This degradation system does not require modification of endogenous proteases, functions robustly over a wide range of adaptor concentrations, and does not require new synthesis of adaptors or proteases to initiate degradation. We demonstrate the efficacy of this system in *E. coli* by degrading tagged variants of LacI repressor and FtsA, an essential cell-division protein. In the latter case, addition of rapamycin causes pronounced filamentation because daughter cells cannot divide. Strikingly, washing rapamycin away reverses this phenotype. Our system is highly modular, with clearly defined interfaces for substrate binding, protease binding, and adaptor assembly, providing a clear path to extend this system to other degradation tags, proteases, or induction systems. Together, these new reagents should be useful in controlling protein degradation in bacteria.



Targeted proteolysis provides a mechanism to control intracellular protein levels in a dynamic fashion and has applications in basic science and biological engineering.^{1–6} Indeed, the perturbation of protein stability has proven critical in the generation of synthetic cellular circuits and has been utilized to investigate loss-of-function phenotypes,^{6–10} providing an alternative and often complementary mechanism to transcriptional knockdowns. A proteolysis-based approach is particularly valuable when pre-existing proteins are inherently long-lived, as terminating the synthesis of such molecules results in relatively slow elimination of the gene product.^{11–16} In these cases, proteins are diluted *via* cell growth and division, often requiring many cell cycles to reach levels that are low enough to eliminate function. During this time period, cells may up-regulate compensatory pathways or acquire suppressor mutations, often obscuring the phenotype of the original perturbation.

When combined with temporal control, targeted proteolysis can be used to investigate essential proteins under control of their native transcriptional and translational control elements. Temperature-sensitive (*ts*) alleles share many of the advantages of targeted proteolysis, but tightly regulated *ts*-alleles are difficult to isolate for many genes. Furthermore, a temperature shift can result in unintended changes in cellular physiology, complicating interpretation. In yeast, for example, shifting the temperature from 25 to 37 °C alters the expression of nearly 1000 genes, half of which have unknown function.¹⁷ Small-molecule inhibitors/activators allow perturbations of native gene function with rapid temporal control under many environmental conditions.¹³ Unfortunately, the identification of highly specific, cell-permeable

inhibitors is not trivial, and conclusively ruling out “off-target” effects is a substantial challenge.

In *Escherichia coli* and most bacteria, processive intracellular proteolysis is mediated by a group of energy-dependent AAA+ proteases, including ClpXP.¹⁸ The ClpXP enzyme degrades substrates *via* a multistep process, which begins with the recognition of an *ssrA* tag or other short peptide sequences, which are exposed in native protein substrates.^{19,20} After ClpX binds a substrate, cycles of ATP hydrolysis drive translocation of the degradation tag through a narrow axial pore, producing a transient unfolding force once the native protein collides with the pore entrance.^{21–23} These pulling events eventually result in global substrate unfolding, allowing translocation of the denatured polypeptide into the lumen of ClpP, where it is cleaved into short peptide fragments.^{24,25} Although degradation rates differ between substrates, ClpX can unfold proteins with a wide range of thermodynamic stabilities and appears to have little sequence specificity in terms of substrate translocation.^{24,26,27} Thus, substrate selectivity is determined by the efficiency of the initial binding event, which can be modulated by accessory factors called adaptors. For example, the SspB adaptor improves ClpXP degradation of *ssrA*-tagged substrates by binding both to the protease and to a portion of the *ssrA* tag, thereby increasing the effective concentration of the substrate relative to the enzyme.^{28–31} Indeed, using synthetic degradation components, we found that tethering alone is sufficient for efficient substrate delivery by SspB.²

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Adaptor proteins have proven useful in engineering controlled degradation systems. For example, McGinness *et al.*⁶ showed that ClpXP degradation became almost entirely dependent on SspB when the C-terminal residues of the *ssrA* tag were mutated from LAA to DAS. Importantly, model substrates bearing DAS tags were selectively degraded in cells when transcription of the *sspB* gene from a *lac* promoter was induced using IPTG.^{4,6} Combining a genetically encoded degradation tag and small-molecule inducer has many of the advantages of classical genetics and pharmacology and can be applied to almost any protein target with temporal control provided by the presence or absence of the small molecule.

In the work reported here, we have engineered and characterized a new targeted degradation system in which the assembly and thus activity of a split adaptor is controlled by the small-molecule rapamycin. Experiments with purified components *in vitro* and with multiple protein substrates in *E. coli* show that ClpXP-mediated degradation of appropriately tagged proteins can be controlled in a rapamycin-dependent manner. This system is simple and generally applicable, requires few genomic modifications, and degrades substrates without the need for new protein synthesis. Moreover, this system should be relatively straightforward to port to other ClpXP-containing bacteria such

as *Caulobacter crescentus*, *Bacillus subtilis*, and *Mycobacterium tuberculosis*.^{6,10,32}

RESULTS AND DISCUSSION

Rapamycin-Dependent Control of Adaptor Function *in Vitro*. We used a DAS+4 *ssrA* tag (AANDENYSENYADAS) to target substrates for adaptor-mediated degradation by ClpXP.⁴ The underlined residues in this tag sequence bind SspB^{CORE} (the folded domain of SspB), whereas the bold residues interact with ClpXP. We split the functionally important portions of SspB into two components, one consisting of SspB^{CORE} fused to FRB, the rapamycin-binding domain of mTOR, and another consisting of the highly flexible ClpX-binding tail of SspB (XB) fused to the FKBP12 protein (Figure 1). Normally, these split components (SspB^{CORE}-FRB and FKBP12-SspB^{XB}) should not interact or deliver substrates to ClpXP. However, rapamycin-dependent dimerization of the FRB and FKBP12 portions of these split proteins^{33–35} should drive assembly of a functional adaptor that delivers DAS+4-tagged substrates to ClpXP for degradation. We verified these expectations by incubating a GFP-DAS+4 substrate with ClpXP protease, FKBP12-SspB^{XB}, SspB^{CORE}-FRB, and an ATP-regenerating system in either the presence or absence of rapamycin. Importantly, no substantial degradation was observed without rapamycin, but robust degradation occurred when rapamycin was present (Figure 2a).

Many molecules must interact for efficient degradation in our system, raising the possibility that slow assembly could limit the degradation rate. To test this possibility, we preincubated substrate, ClpXP, and the adapter components and monitored the kinetics of GFP-DAS+4 degradation before and after addition of rapamycin (Figure 2b). After adding rapamycin, steady-state degradation was reached within 20 s, the dead-time of the experiment. Thus, rapamycin-dependent formation of the active proteolysis complex occurs on a reasonably fast time scale.

Next, we determined how degradation efficiency varied with the stoichiometry of different components. First, we titrated a mixture containing equal amounts of the two split-adaptor components against a fixed concentration of protease, substrate, and rapamycin. Degradation increased in a roughly hyperbolic fashion (Figure 3a), with half-maximal stimulation at $\sim 0.5 \mu\text{M}$

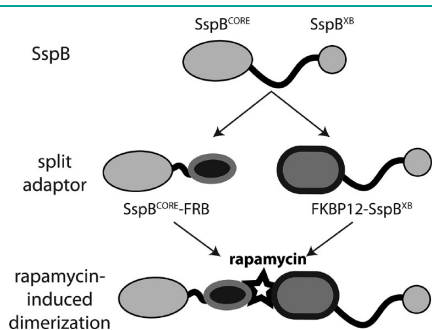


Figure 1. Cartoon of the split adaptor. The SspB core domain (residues 1–113) was split from the ClpX-binding tail (SspB^{XB}, residues 139–156). SspB^{CORE} was fused to FRB, and FKBP12 was fused to SspB^{XB}. Addition of rapamycin reconstitutes a complete adaptor.

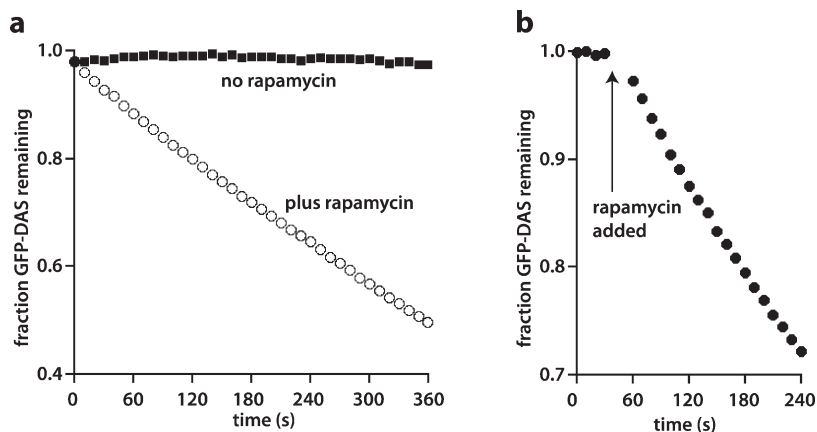


Figure 2. Rapamycin-dependent degradation. (a) Incubation of GFP-DAS+4 ($2 \mu\text{M}$), ClpX₆ ($0.3 \mu\text{M}$), ClpP₁₄ ($0.9 \mu\text{M}$), FKBP12-SspB^{XB} ($5 \mu\text{M}$), SspB^{CORE}-FRB ($5 \mu\text{M}$), and rapamycin ($8 \mu\text{M}$) resulted in GFP-DAS+4 degradation at a rate of $0.58 \text{ min}^{-1} \text{ enzyme}^{-1}$. No degradation was observed in the absence of rapamycin. (b) Addition of rapamycin resulted in rapid assembly of the degradation complex. GFP-DAS+4 ($2 \mu\text{M}$), ClpX₆ ($0.3 \mu\text{M}$), ClpP₁₄ ($0.9 \mu\text{M}$), FKBP12-SspB^{XB} ($5 \mu\text{M}$), and SspB^{CORE}-FRB ($5 \mu\text{M}$) were preincubated at 30°C . Rapamycin ($8 \mu\text{M}$) was added at the time indicated by the arrow. Note that the y-axis does not extend to 0 in both panels.

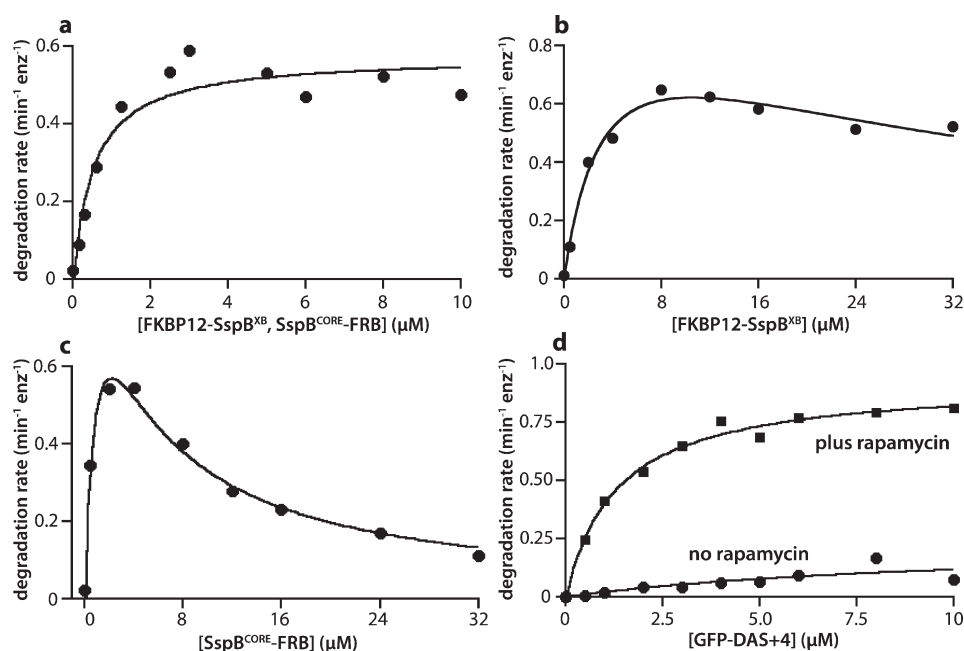


Figure 3. Dependence of GFP-DAS+4 degradation on adaptor or substrate concentration. (a) Degradation in the presence of increasing equimolar concentrations of FKBP12-SspB^{XB} and SspB^{CORE}-FRB. The solid line is a hyperbolic fit with an apparent binding constant of 0.51 μM . (b) Degradation in the presence of a fixed concentration of SspB^{CORE}-FRB (5 μM) and increasing FKBP12-SspB^{XB}. Data were fit to eq 1, where $k_1 = 1.1 \text{ min}^{-1} \text{ enz}^{-1}$, $K_a = 3.4 \mu\text{M}$, and $K_b = 33 \mu\text{M}$. (c) Degradation with a fixed concentration of FKBP12-SspB^{XB} (5 μM) and increasing concentrations of SspB^{CORE}-FRB. Data were fit using eq 1 with parameters $k_1 = 1.2 \text{ min}^{-1} \text{ enz}^{-1}$, $K_a = 1.3 \mu\text{M}$, and $K_b = 3.9 \mu\text{M}$. (d) Substrate dependence of degradation in the presence of SspB^{CORE}-FRB (5 μM) and FKBP12-SspB^{XB} (5 μM). Fitting the rapamycin data to the Michaelis–Menten equation gave $K_M = 1.3 \mu\text{M}$ and $V_{\text{max}} = 0.92 \text{ min}^{-1} \text{ enz}^{-1}$. In all panels, the concentrations of ClpX₆ and ClpP₁₄ were 0.3 and 0.9 μM , respectively. In panels a–c and the “plus rapamycin” experiment of panel d, the rapamycin concentration was 12 μM . In panels a–c, the GFP-DAS+4 concentration was 2 μM .

SspB^{CORE}-FRB and FKBP12-XB. Second, we added increasing concentrations of FKBP12-XB to fixed quantities of all other components and observed half-maximal stimulation at 2–4 μM FKBP12-XB (Figure 3b). Third, we titrated increasing SspB^{CORE}-FRB against fixed concentrations of the other components (Figure 3c). Degradation increased initially and then was inhibited at high SspB^{CORE}-FRB concentrations (Figure 3c), probably because free SspB^{CORE}-FRB competes with the limited number of SspB^{CORE}-FRB·rapamycin·FKBP12-XB complexes for substrate binding. Finally, we measured degradation as the substrate concentration was varied in the presence or absence of rapamycin using fixed concentration of protease and the split-adaptor proteins (Figure 3d). As expected, slow degradation was observed in the absence of rapamycin and robust degradation was observed with rapamycin present. In combination, these results show that many enzyme and adaptor concentrations mediate efficient rapamycin-dependent degradation. For maximum efficiency, however, the ratio of FKBP12-XB to SspB^{CORE}-FRB should be equal to or greater than 1.

$$\text{rate} = \frac{k_1 [\text{SspB}^{\text{CORE}}\text{-FRB}]}{K_a + [\text{SspB}^{\text{CORE}}\text{-FRB}] + \frac{[\text{SspB}^{\text{CORE}}\text{-FRB}]^2}{K_b}} \quad (1)$$

Controlled Degradation of a Transcriptional Repressor.

Given the promising results *in vitro*, we developed an assay for rapamycin-dependent degradation *in vivo*. To prevent uncontrolled substrate delivery and degradation, the *sspB* gene was

deleted from the chromosome of *E. coli* strain W3110. Next, we introduced a DAS+4 tag at the C-terminus of the *lacI* transcriptional repressor, using a scar-less λ -RED-based recombination technique, and transformed this strain with a plasmid (pJD427) bearing constitutive promoters driving production of FKBP12-XB and SspB^{CORE}-FRB.³⁶ We expected LacI-DAS+4 to repress *lacZ* transcription and thus production of β -galactosidase in the absence of rapamycin (Figure 4a), with addition of rapamycin resulting in LacI-DAS+4 degradation and induction of *lacZ* expression.

In vitro, we observed rapamycin-dependent degradation of LacI-DAS+4 using ClpXP protease, FKBP12-SspB^{XB}, SspB^{CORE}-FRB, and an ATP-regenerating system (Figure 4b). To assay for LacI-DAS+4 degradation *in vivo*, we grew strain JD704 (W3110 *lacI*-DAS+4 *sspB*/pJD427) in M9 medium at 37 °C to mid phase and added either rapamycin (growth of the W3110 parent strain was unchanged from 0 to 50 μM rapamycin; not shown), IPTG as a positive control for induction, or an equal volume of DMSO (the solvent for rapamycin and IPTG) as a negative control. At various times, samples were taken and β -galactosidase activity was assayed and normalized to OD₆₀₀. Addition of either rapamycin or IPTG led to increased β -galactosidase activity, but DMSO had no effect (Figure 4c). Rapamycin-dependent induction of *lacZ* mRNA levels in the LacI-DAS+4 strain was also observed by RT-qPCR, confirming a direct effect on transcription (data not shown). Compared with IPTG induction, rapamycin induction in the LacI-DAS+4 strain showed a lag and somewhat slower kinetics (Figure 4c). This lag may result from slow diffusion of the drug into the cell or from relatively slow ClpXP-mediated degradation. We note, however, that

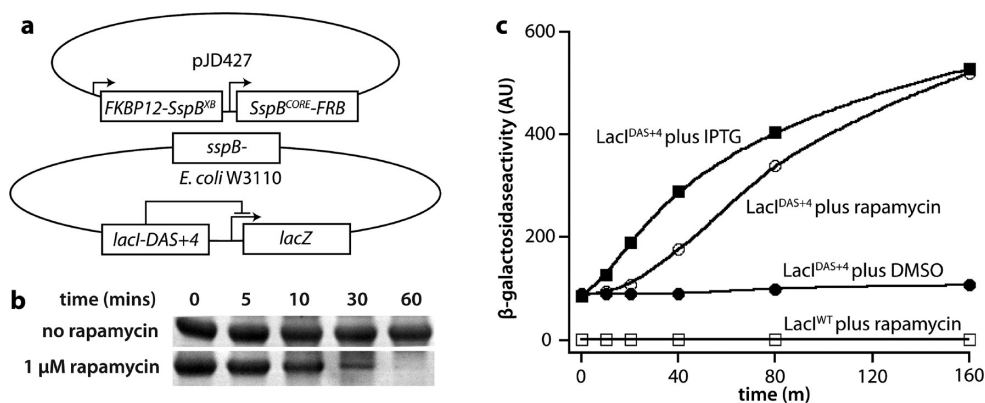


Figure 4. Degradation of LacI-DAS+4. (a) Assay strains contained an *sspB* deletion and a DAS+4 tag at the C-terminus of LacI, which represses transcription of *lacZ*. Production of the split-adaptor components is mediated by plasmid-borne constitutive promoters. (b) Time course of degradation of LacI-DAS+4 (8 μ M) by ClpX₆ (0.3 μ M), ClpP (0.9 μ M), SspB^{CORE}-FRB (5 μ M), FKBP12-SspB^{XB} (5 μ M), and 10 μ M rapamycin (when present). (c) β -Galactosidase activities were measured in strains containing LacI-DAS+4 following addition of rapamycin (10 μ M), IPTG (5 mM), or DMSO. An isogenic strain expressing wild-type LacI showed no response to rapamycin. In the absence of inducer, the LacI-DAS+4 strain exhibited increased basal β -galactosidase activity relative to the wild-type LacI strain. Further experiments indicated that the observed derepression is not solely due to rapamycin-independent LacI-DAS+4 degradation or SspB^{CORE}-FRB mediated inhibition of LacI activity.³⁷

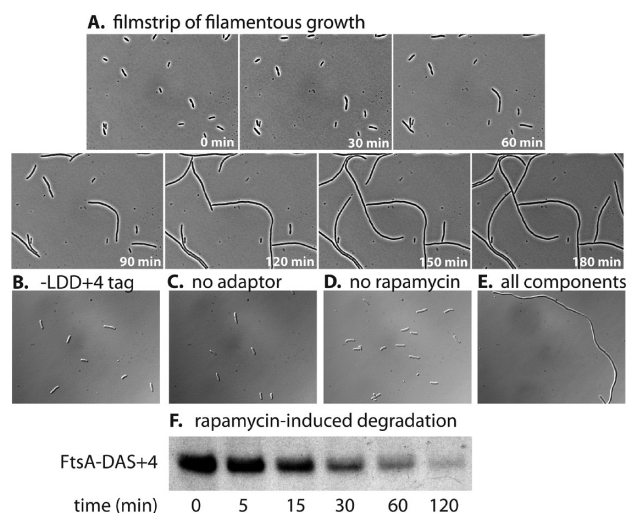


Figure 5. Rapamycin-dependent degradation of an essential cell division protein. (A) *E. coli* W3110 *sspB*-bearing plasmid pJD427 was modified at the *ftsA* loci by addition of a DAS+4 tag fused to a kanamycin marker (JD784). This strain was grown in medium lacking rapamycin before cells were mounted on a microscope slide bearing LB/agar/rapamycin (10 μ M) and imaged every 30 min. (B) *E. coli* W3110 *sspB*⁻ / pJD427 with a nondegradable LDD+4 tag fused to FtsA exhibited normal cell morphology when grown in the presence of 10 μ M rapamycin. Strain JD784 lacking the adaptor system (C) or grown in medium lacking rapamycin (D) also exhibited normal cell morphology. (E) Addition of both pJD427 and 10 μ M rapamycin to strain JD784 resulted in a strong filamentation phenotype. (F) Western blotting shows intracellular degradation of FtsA-DAS+4 after addition of rapamycin (10 μ M) in *E. coli* W3110 *sspB*⁻ / pJD427. Protein synthesis was not blocked in this experiment. The sample volume in each lane was adjusted to contain the same amount of total cellular protein.

rapamycin-dependent induction still occurred on the time scale of many biological processes. Relative to wild-type strains, LacI-DAS+4 strains exhibited elevated β -galactosidase activity, even in the absence of rapamycin. Control experiments revealed that this derepression resulted from a combination of effects including disruption of the *lac* operon by introduction of the

tag-coding sequence, as well as by adaptor- and rapamycin-independent effects on LacI-DAS+4 repressor activity.³⁷

Regulated Proteolysis of an Essential Cell-Division Protein. Regulated proteolysis obviates the need to alter synthesis of a protein of interest. Unlike genetically encoded knockouts, or techniques reliant on a regulated promoter, our approach leaves the natural regulatory components in place. This could be important for studying genes with complex regulatory patterns, such as those involved in cell-cycle progression or cell division.^{38–43}

To assay the efficacy of our system in controlling a cell-cycle regulated protein, we appended a degradation tag to the C-terminus of the FtsA cell-division protein. FtsA is a membrane-associated ATP-binding protein that interacts with FtsZ (the Z-ring filament-forming protein) and recruits downstream cell-division proteins.⁴⁴ Previous studies using depletion strains and temperature-sensitive *ftsA* mutants have shown that loss of FtsA function results in incomplete septation and long, filamentous cells.^{45–47} We integrated a sequence encoding a DAS+4 tag at the 3' end of the chromosomal copy of *ftsA* in *E. coli* strain W3110 *sspB*⁻ and transformed this strain with plasmid pJD427, encoding the split-adaptor proteins. For control experiments, we also constructed a strain in which FtsA had a C-terminal LDD+4 tag, which allows for SspB^{CORE}-FRB binding but does not target proteins to ClpXP.⁴ After growing this strain in M9 medium, cells were spotted on an M9-agarose pad with 10 μ M rapamycin, pressed between two microscope slides, and then imaged as a function of time. Over the course of the experiment, cells formed long filaments, consistent with efficient proteolysis of FtsA-DAS+4 (Figure 5A). To ensure that this phenotype depended on degradation, variants were grown in M9 medium to m phase (OD 0.7), diluted 30-fold into the same medium with or without 10 μ M rapamycin, and grown for an additional 2 h at 37 °C. At this time, cell morphology was observed using phase-contrast microscopy. Only strains grown in the presence of rapamycin, containing pJD427, and harboring a DAS+4 tagged copy of *ftsA* displayed a filamentation phenotype (Figure 5B–E). To determine the efficiency of rapamycin-dependent degradation *in vivo*, we prepared cell lysates at various times after treatment with rapamycin and subjected them to SDS-PAGE and Western blotting

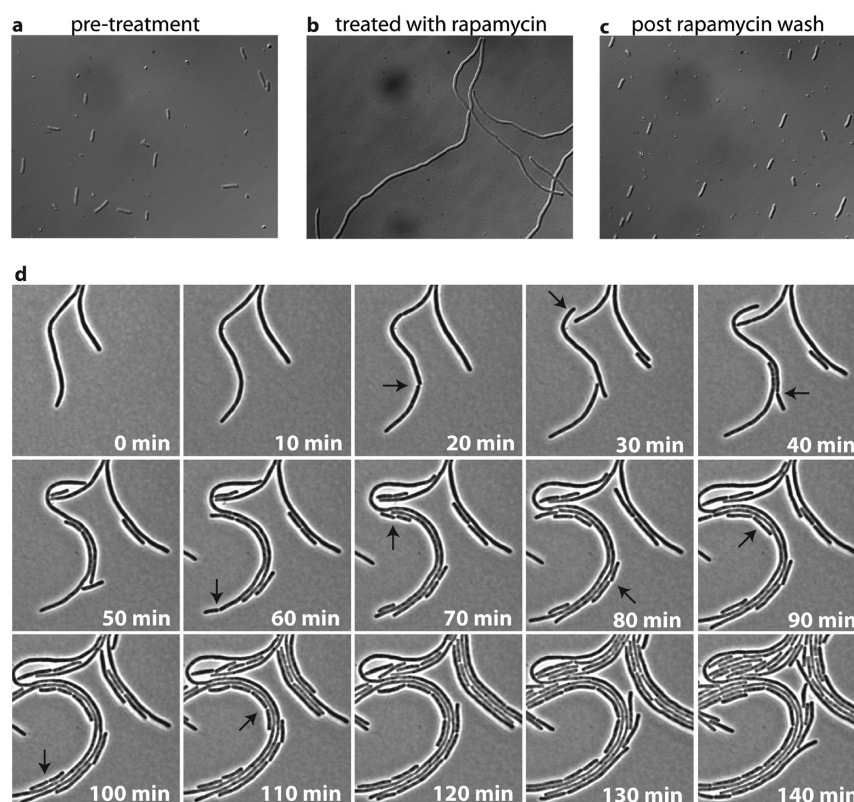


Figure 6. Rapamycin-dependent filamentation is reversible. (a) JD784 cells exhibit normal morphology before treatment with rapamycin. (b) After growth in the presence of $10\ \mu\text{M}$ rapamycin, strong filamentation was observed. (c) Growth of filamentous cells in rapamycin-free medium results in the appearance of cells with normal morphology. (d) Filamentous cells grown in the presence of rapamycin were harvested and washed to remove rapamycin, immobilized in LB/agar on a microscope slide, and imaged every 10 min using an inverted microscope (the initial image was taken 10–15 min after beginning the washing procedure). Arrows indicate the formation of septa in dividing cells.

using an anti-FtsA antibody (a generous gift of Dr. Miguel Vicente, Centro Nacional de Biotecnología, Madrid, Spain). Although protein synthesis continued, the steady-state level of FtsA decreased rapidly with a half-life of ~ 15 min (Figure 5F). These results indicate that degradation is efficient and depends on the small-molecule inducer, the genetically encoded degradation tag, and the split-adaptor system.

Because degradation depends on rapamycin, we tested if filamentation could be reversed by washing out the small molecule. Thus, we grew cells in M9 medium containing rapamycin to mid-log phase (Figure 6a,b), harvested cells by centrifugation, washed with rapamycin-free medium, and then resuspended cells and diluted them 40-fold into rapamycin-free M9 medium. After growth for 3 h at $37\ ^\circ\text{C}$, cells appeared normal and were not filamentous (Figure 6c). In principle, this rescue could arise from multiple rounds of division by the filamentous cells or by the death of these cells and growth of a minority of nonfilamentous cells in the original culture. To distinguish between these possibilities, we imaged live cells after removal of rapamycin. Strain JD784 (W3110 *sspB*⁻, *ftsA::ftsA-DAS+4*, pJD427) was grown in M9 medium supplemented with rapamycin to OD 0.5. Cells were harvested, washed with medium lacking rapamycin, spotted on an M9-agarose pad, and placed between microscope slides for imaging. Importantly, this experiment revealed the formation of multiple septa throughout individual filamentous cells and consequent division, indicating that removal of rapamycin allowed these cells to accumulate FtsA-DAS+4 and to restart successful cell division within an hour of rapamycin removal (Figure 6d).

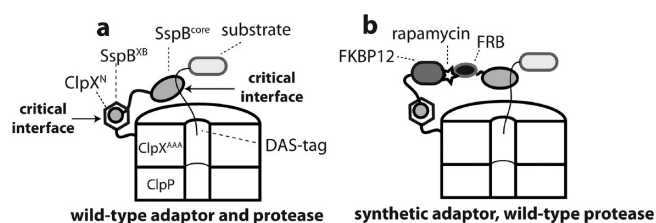


Figure 7. Split adaptor systems preserve critical protein-binding interfaces. (a) Substrate delivery by wild-type SspB requires simultaneous binding between the XB tails of the adaptor and the N-domain of ClpX and between the substrate-binding domain of SspB and a *ssrA* tag on the substrate. (b) These critical interfaces are preserved in our engineered system, but formation of a complete adaptor is under control of rapamycin, a small-molecule inducer of dimerization.

Discussion. Adaptor proteins must be capable of binding substrates and also tethering the bound substrate to a protease (Figure 7a). In the wild-type SspB adaptor, these functions reside in a core substrate-binding domain and an unstructured C-terminal tail, respectively.^{29–31,48} The results presented here show that these adaptor functions can reside in separate fusion proteins, with adaptor assembly and function depending on the presence of rapamycin, which mediates dimerization of the FRB and FKBP12 domains of the split components (Figure 7b). This split-adaptor system allows rapamycin-dependent degradation of appropriately tagged substrates by the wild-type ClpXP protease both *in vitro* and *in vivo*. Our results also provide a framework for

extending the system. For example, it should be straightforward to change substrate specificity by exchanging the substrate-binding domain from *E. coli* SspB with a different peptide-binding domain. The modularity of our adaptor design may also facilitate the incorporation of other dimerization domains. Indeed, recent reports have demonstrated spatiotemporal control of protein dimerization using light.^{49–51} Replacing the FKBP12 and FRB domains in our split adaptor system with such a pair (e.g., PhyB and PIF) could result in a light-induced protein degradation system affording both spatial and temporal control.

Critically, our split-adaptor system combines the advantages of small-molecule control with utilization of the endogenous ClpXP protease. Thus, there is no requirement for introduction of an exogenous protease, which might degrade unintended substrates. Moreover, our system was effective over a wide-range of component stoichiometries, simplifying its implementation *in vivo*. ClpXP is present in many bacteria, and *E. coli* SspB has been shown to deliver substrates to ClpX homologues from *C. crescentus*, *B. subtilis*, and *M. tuberculosis*.^{6,10,32} Porting the split-adaptor system to these or other bacterial species should be as simple as introducing a plasmid expressing roughly comparable amounts of the two adaptor components and adding an appropriate degradation tag to the protein target of interest. We found that a split-adaptor system using some parts of *C. crescentus* SspB and modified *C. crescentus* degradation tags worked well *in vitro*, in a manner that was not affected by the presence of *E. coli* SspB.³⁷ This ability to interchange modular components should prove useful in optimizing split-adaptor systems with altered degradation-tag specificity.

We have shown that modification of the wild-type LacI repressor by addition of a C-terminal DAS+4 tag allows rapamycin-dependent degradation and induction of β -galactosidase expression. The kinetics of β -galactosidase induction using rapamycin were slower than those observed using IPTG, a small molecule that binds directly to LacI repressor and reduces its affinity for operator DNA \sim 1000-fold.⁵² Rapamycin may enter cells more slowly than IPTG, or the rate of rapamycin-mediated induction may be limited by other factors, such as the rate of repressor dissociation from the operator or the rate of ClpXP degradation. Importantly, however, similar final levels of β -galactosidase expression were observed for both small-molecule inducers indicating near complete degradation of LacI repressor by our system. Other transcription factors may also be potential targets for rapamycin-dependent induction. For example, we found that modification of the tetracycline repressor (derived from transposon Tn10) with a C-terminal DAS+4 tag resulted in rapamycin-dependent ClpXP degradation *in vitro* (J.H. Davis, unpublished).

Finally, we have demonstrated that an essential cellular protein, which is subject to complex transcriptional and post-transcriptional regulation, can be studied using our proteolysis-based knockdown system. After fusing the DAS+4 tag to the cell-division protein FtsA, we observed pronounced filamentation upon induction of the degradation system using rapamycin. Importantly, filamentation was completely dependent on the presence of the DAS+4 tag, the adaptor system, and rapamycin. Unlike a genetically encoded knockout, our small-molecule-dependent system is reversible. After removing rapamycin from the culture medium, we observed the division of filamentous cells, eventually returning to normal cell morphology.

Rapamycin-dependent assembly of split adaptors has many potential applications. Because this system does not require synthesis of

new adaptor proteins,^{4,6,10} degradation could be initiated at the same time as inhibition of transcription or translation, allowing for faster clearance of target proteins from the cell. Additionally, this system, which relies on the pre-existing pool of adaptors, could be used to directly study proteins involved in transcription and translation. Indeed, Moore *et al.*³ demonstrated that ClpXP could forcefully extract a ribosomal protein from an intact 50S particle *in vitro*. Split-adaptor degradation systems might allow similar experiments *in vivo*, helping to elucidate the physiological function of essential genes required for transcription and translation.

METHODS

Plasmids and Strains. GFP variants used in this study contained the sequence H₆-IDDLG at the N-terminus in addition to the mutations S2R, S65G, S72A, and M78R. The sequences of the DAS+4 (AANDENYSENYADAS) and LDD+4 (AANDENYSENYALDD) tags were appended directly to the C-terminus of substrates.

LacI degradation experiments were performed in *E. coli* strain W3110 *sspB*[−] with the degradation tag (DAS+4) introduced at the C-terminus of LacI repressor as described below. Strains also contained pJD427, which drives constitutive production of the split adaptor components, SspB^{CORE}-FRB from the weak proB promoter and FKBP12-SspB^{XB} from the strong proC promoter;³⁶ these promoter strengths were chosen to increase the chance that FKBP12-SspB^{XB} would be in excess of SspB^{CORE}-FRB, a condition that results in robust delivery *in vitro* (Figure 3b,c). The pJD427 plasmid bears a medium-copy p15a origin of replication and also expresses chloramphenicol acetyltransferase. For each split-adaptor gene, transcription was terminated using the Bba_B0011 element and translation was initiated using the Bba_B0032 ribosome-binding site (for descriptions of these sequences, see www.partsregistry.org).

FtsA-depletion studies were performed in *E. coli* W3110 *sspB*[−] bearing pJD427. A DAS+4 degradation tag fused to a kanamycin-resistance marker was introduced at the C-terminus of the FtsA coding sequence *via* λ -RED mediated recombination using primers *ftsA-a*, *ftsA-b* (Table 1).

Scar-less λ -RED-Mediated Chromosomal Manipulation. The λ -RED-mediated recombinering machinery, which is encoded on the plasmid vector pSIM5, was used to integrate degradation tags into the chromosome and to knockout *sspB*.⁵³ Scar-less genomic manipulation was achieved by first introducing a cassette encoding both selectable and counter-selectable markers. Successful recombinants were identified using the selectable marker, the entire cassette was then targeted for replacement, and recombinants were identified using the second counter-selectable marker.

Our targeting cassette contained a constitutive promoter, (Bba_J23116) and ribosome-binding site (Bba_B0032; www.partsregistry.org), used to drive production of our counter-selectable marker, mPheS as well as a kanamycin-resistance marker (Bba_P1003). mPheS, a mutant variant of *E. coli* phenylalanine tRNA synthetase (mPheS), incorporates ρ -chlorophenylalanine (ρ -Cl-Phe) into cellular tRNA and proteins and is lethal when cells are grown in the presence of ρ -Cl-Phe.^{54–57}

The mPheS-kan^R cassette was PCR amplified from plasmid pJD141, using primers with 20 bp of homology to the cassette and 40 bp of homology to target either the *sspB* ORF (primers *sspB-a*, *sspB-b*, Table 1) or the 3' terminus of *lacI* (primers *lacI-a*, *lacI-b*, Table 1). Plasmid DNA was removed by restriction digestion with DpnI, followed by gel purification of the PCR product.

After induction of the λ -RED recombination proteins by heat shock for 15 min at 42 °C, PCR products (100 ng) were electroporated into cells, which were then allowed to recover for 6 h at 30 °C, before plating on LB/kanamycin (20 μ g mL^{−1}) at 30 °C. Successful recombinants exhibited resistance to 20 μ g mL^{−1} kanamycin and sensitivity to 16 mM

Table 1. Recombineering Primers^a

<i>sspB</i> -a	AAGCAGAACGTGAAATGCGTCTGGGCCGGAGTTAATCTGTgaattcgcggccgcttag
<i>sspB</i> -b	CATTA AAAAGACAAAACAGGCCCGCTGGGCCTGTTTTGTActgcagcggcgctactagt
<i>lacI</i> -a	GCAGCTGGCAGCACAGGTTTTCCCGACTGGAAAGCGGGCAGgaattcgcggccgcttag
<i>lacI</i> -a	TGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCctgcagcggcgctactagt
<i>sspB</i> -ko-1	AAGCAGAACGTGAAATGCGTCTGGGCCGGAGTTAATCTGTactgattgtgtgaagtaa
<i>sspB</i> -ko-2	CATTA AAAAGACAAAACAGGCCCGCTGGGCCTGTTTTGTAttactcacaacaatcagt
<i>lacI</i> -DAS-1	GCAGCTGGCAGCACAGGTTTTCCCGACTGGAAAGCGGGCAGgcagcctaactgaaacta
<i>lacI</i> -DAS-2	TGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCctgcagcggcgctactagt
<i>ftsA</i> -a	GTGGATCAAGCGACTCAATAGTTGGCTGCGAAAAGAGTTTTgcagcctaactgaaacta
<i>ftsA</i> -b	CTGTGCGCTGAGGCCGTAATCATCGTTCGGCCTCATAAAAAttattagaaaactcatcga

^aResidues underlined are used to form duplexes with the target sequence. Residues in uppercase font are homologous to the appropriate *E. coli* chromosomal locus.

ρ -Cl-Phe and were verified by colony-PCR. dsDNA cassettes bearing the desired insertion sequence (flanked by the same 40 bp overhangs described above) were prepared by PCR using primers *sspB*-ko-1, *sspB*-ko-2 (generating *sspB*⁻) or *lacI*-DAS-1, *lacI*-DAS-2 (resulting in *lacI*-DAS+4). Cassettes were electroporated into cells prepared for recombination and were allowed to recover at 30 °C for 6 h before plating on YEG-agar/16 mM ρ -Cl-Phe at 30 °C (YEG-agar consists of 0.5% yeast extract, 1% NaCl, 0.4% glucose, 1.5% agar). Successful replacement of the mPheS-kan^R cassette was verified by PCR amplification and sequencing of the region of interest. pSIMS was cured from the cells *via* serial dilution and growth at 30 °C under nonselective conditions.

Protein Purification. *E. coli* ClpX, *E. coli* ClpP, *E. coli* SspB, and GFP-DAS+4 were expressed and purified as described.^{2,4,58} FKBP12-SspB^{XB} was expressed with an N-terminal thrombin-cleavable His₆ tag from a pET28 vector in *E. coli* BLR (F⁻, *ompT*, *gal*, *dcm*, *lon*, *hdsS*_B(r_B⁻ m_B⁻), λ (DE3), *recA*⁻). Cells were grown at RT in 1.5YT broth (1.3% tryptone, 0.75% yeast extract, and 0.75% NaCl, [pH 7.0]) to OD₆₀₀ 0.7, induced with 1 mM IPTG, and harvested by centrifugation 4 h after induction. Cell pellets were resuspended in LB1 buffer (20 mM HEPES [pH 8.0], 400 mM NaCl, 100 mM KCl, 20 mM imidazole, 10% glycerol, and 10 mM 2-mercaptoethanol) and lysed by addition of 1 mg mL⁻¹ lysozyme followed by sonication. Benzonase was added to lysates for 30 min prior to centrifugation at 8000 rpm in a Sorvall SA800 rotor. The supernatant was applied to a Ni²⁺-NTA affinity column (Qiagen), washed with 50 mL of LB1 buffer, and eluted with LB1 buffer supplemented with 190 mM imidazole. Fractions containing FKBP12-SspB^{XB} were pooled and chromatographed on a Sephacryl S-100 gel filtration column in GF-1 buffer (50 mM Tris-HCl [pH 7.6], 1 mM dithiothreitol, 300 mM NaCl, 0.1 mM EDTA, and 10% glycerol). Fractions were analyzed by SDS-PAGE, concentrated to 1 mL, and incubated with thrombin overnight at RT. Cleaved FKBP12-SspB^{XB} was purified away from thrombin using a S100 gel-filtration column. Analysis by SDS-PAGE confirmed complete cleavage. FKBP12-SspB^{XB} was concentrated and stored at -80 °C.

SspB^{CORE}-FRB contained an internal His₆ tag separating the two domains and was expressed from a pACYC-derived vector in *E. coli* BLR as described for FKBP12-SspB^{XB}. Harvested cells were resuspended in LB2 buffer (100 mM NaH₂PO₄ [pH 8.0], 10 mM Tris-HCl, 6 M GuHCl, 300 mM NaCl, and 10 mM imidazole) and stored at -80 °C prior to purification. Cells were lysed by rapidly thawing the cell pellet, followed by centrifugation as described above. The supernatant was applied to a Ni²⁺-NTA column, washed with 50 mL LB2 buffer, and eluted with LB2 buffer supplemented with 240 mM imidazole. After overnight dialysis against GF2 buffer (20 mM Tris-HCl [pH 8.0], 10% glycerol, 25 mM NaCl, 25 mM KCl, 1 mM dithiothreitol), soluble protein was chromatographed on a Sephacryl S100 gel-filtration column. Fractions were analyzed by SDS-PAGE, concentrated, and stored

at -80 °C. LacI-DAS+4 containing a cleavable His₆ tag was purified by Ni²⁺-NTA affinity chromatography, tag cleavage, and gel-filtration chromatography as described above.

β -Galactosidase Activity Assays. To measure β -galactosidase activity, strains were grown in 1 mL of supplemented M9 medium (M9 salts, 1 mM thiamine hydrochloride, 0.2% casamino acids, 2 mM MgSO₄, 0.1 mM CaCl₂, 0.4% glycerol, and 35 μ g mL⁻¹ chloramphenicol when appropriate) at 37 °C in aerated culture vials. At mid-log phase, the OD₆₀₀ was measured, an aliquot (20 μ L) was taken for a Miller assay, and the cells were treated with either rapamycin (10 μ M), IPTG (5 mM), or an equal volume of DMSO (the solvent for both IPTG and rapamycin). The final DMSO concentration in each sample was 0.5%. Growth of this strain was unaffected by DMSO concentrations up to 4% (data not shown). At different times after treatment, OD₆₀₀ was measured (150 μ L) in a SpectraMax plate reader (Molecular Devices), and samples (20 μ L) were quenched by adding 80 μ L of Z-lysis buffer (B-PERII (Pierce) supplemented with 200 μ g mL⁻¹ spectinomycin and 1 mM PMSF). One milliliter of Z-assay buffer (66 mM Na₂PO₄ [pH 7.4], 6 mM KCl, 700 μ M MgCl₂, 1 mM DTT, and 0.67 mg mL⁻¹ *o*-nitrophenyl β -d-galactoside) was then added to each sample, before aliquoting samples (150 μ L) into a 96-well plate and measuring absorbance at 420 nm as a function of time using a SpectraMax plate reader (Molecular Devices). Sample OD₆₀₀ was corrected for path length using the equation

$$\text{OD}_{600} = (\text{OD}_{600\text{measured}} - 0.04) \times 3.39$$

For each sample and time point, β -galactosidase activity was reported as

$$\beta\text{-gal activity} = 10,000 \times \frac{\Delta A_{420}}{\Delta \text{time (mins)} \times \text{OD}_{600}}$$

Degradation Assays. Degradation assays *in vitro* were performed in PD buffer (25 mM HEPES KOH [pH 7.6], 5 mM MgCl₂, 10% glycerol, and 200 mM KCl) at 30 °C. GFP fluorescence was monitored by exciting with 467 nm light and measuring emission at 511 nm using a SpectraMax M5 96-well fluorescence plate reader (Molecular Devices) or a spectrofluorimeter (Photon Technology International). Each degradation reaction contained an ATP-regeneration mix, consisting of 4 mM ATP, 16 mM creatine phosphate, and 0.32 mg mL⁻¹ creatine kinase.⁵⁸ Degradation reactions of LacI-DAS+4 *in vitro* were quenched by boiling in GB buffer (50 mM Tris-HCl [pH 6.8], 75 mM dithiothreitol, 35 mM 2-mercaptoethanol, 0.003% bromophenol blue, 0.015% sodium dodecyl sulfate, 8% glycerol) and resolved by SDS-PAGE. Degradation assays *in vivo* were performed by centrifuging 1 mL of 0.5 OD₆₀₀ cultures, resuspending the pellet in 50 μ L WB (8 M urea, 50 mM Tris-HCl [pH 7.6]), normalizing each sample by total protein

content using a Bradford assay, running SDS-PAGE, transferring by electro-blotting to a PVDF membrane, and probing using the polyclonal anti-FtsA antibody, MVM1 (a gift from Dr. Miguel Vicente, Centro Nacional de Biotecnología, Madrid, Spain).

Microscopy. Live cells were spotted onto M9–1.5% agarose pads (supplemented with 10 μ M rapamycin when applicable) and imaged using an Axiovert 200 microscope (Zeiss) with a 633/1.4 NA objective (Zeiss) fitted with an objective heater (Bioprotechs) maintained at 37 °C.

AUTHOR INFORMATION

Corresponding Author

*E-mail: bobsauer@mit.edu.

Present Addresses

⁵Ginkgo BioWorks, Inc., Boston, MA.

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