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Regulation of Bacterial Gene Expression by Protease-Alleviated Spatial Sequestration (PASS)

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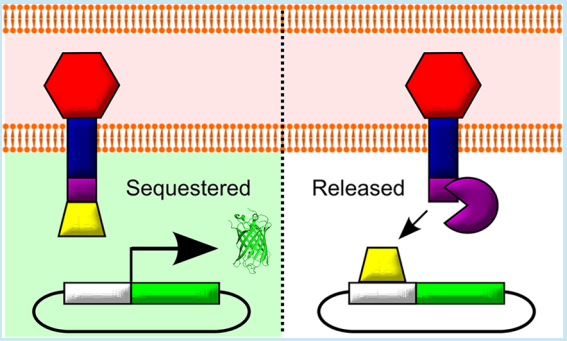
Supporting Information

ABSTRACT: In natural microbial systems, conditional spatial sequestration of transcription factors enables cells to respond rapidly to changes in their environment or intracellular state by releasing presynthesized regulatory proteins. Although such a mechanism may be useful for engineering synthetic biology technologies ranging from cell-based biosensors to biosynthetic platforms, to date it remains unknown how or whether such conditional spatial sequestration may be engineered. In particular, based upon seemingly contradictory reports in the literature, it is not clear whether subcellular spatial localization of a transcription factor within the cytoplasm is sufficient to preclude regulation of cognate promoters on plasmid-borne or chromosomal loci. Here, we describe a modular, orthogonal platform for investigating and implementing this mechanism using protease-alleviated spatial sequestration (PASS). In this system, expression of an exogenous protease mediates the proteolytic release of engineered transcriptional regulators from the inner face of the *Escherichia coli* cytoplasmic membrane. We demonstrate that PASS mediates robust, conditional regulation of either transcriptional repression, *via* tetR, or transcriptional activation, by the λ phage CI protein. This work provides new insights into a biologically important facet of microbial gene expression and establishes a new strategy for engineering conditional transcriptional regulation for the microbial synthetic biology toolbox.



KEYWORDS: *gene regulation, subcellular organization, transcription factor, signaling, microbiology*

Conditional spatial sequestration of transcription factors is a strategy widely employed by prokaryotes to achieve gene regulation. Among the best characterized examples is the *Escherichia coli* mechanism for regulated uptake of maltose.[1,2] In this system, the $MalFGK_2$ maltose transporter spans the cytoplasmic membrane, and in its resting state, this transporter sequesters the transcriptional activator MalT at the cytoplasmic membrane.[3] When MalE binds maltose in the periplasm, this complex binds to the transporter and promotes a series of ATP hydrolysis-linked conformational changes,[4] which eventually result in the release of MalT from the cytoplasmic face of the transporter. MalT then drives downstream *mal* genes in a manner dependent on endogenous maltotriose (a product of glycogen degradation[5]). Thus, in this case, conditional localization of the transcriptional regulator is mediated by conformation-dependent protein−protein interactions.

A distinct mechanism for controlling localization of transcriptional regulators is regulated intramembrane proteolysis (RIP), which is conserved from prokaryotes to eukaryotes.[6] In RIP, the sequential proteolysis of extra- and intramembrane segments of a transmembrane protein leads to the cytoplasmic release of a transcription regulator.[7] One such system is the σ^E stress response in *E. coli*, whereby accumulation of misfolded outer membrane porins is sensed to drive expression of stress response genes, such as chaperones.[8] In this system, σ^E is initially bound to and inhibited by the cytoplasmic membrane antisigma factor, RseA.[9,10] Binding of misfolded porins to RseA renders the periplasmic domain of RseA labile to cleavage by DegS, and this cleavage subsequently renders the cytoplasmic domain of RseA labile to cleavage by YaeL.[11−13] Following this second cleavage event, sequestration of σ^E is alleviated and it diffuses from the membrane to recruit RNA polymerase to promoters of stress-response genes. Thus, in both the σ^E RIP system and the MalT system, conditional control of gene expression is mediated by alleviation of spatial sequestration of a presynthesized transcription factor.

Importantly, these natural systems illustrate that conditional spatial sequestration of transcription factors enables rapid responses to changes in environmental or cellular state, since

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such responses do not require novel synthesis of transcriptional regulators to coordinate downstream gene expression. Thus, spatial conditional sequestration may also be useful for engineering synthetic biology technologies ranging from biosensing to coordination of engineered metabolic pathways. However, to date it remains unclear how or whether conditional spatial sequestration may be engineered. In some cases, artificial sequestration of native transcriptional regulators has proven sufficient to mediate spatial control of transcription. For example, targeting the transcriptional repressor Mlc to the inner face of the *E. coli* cytoplasmic membrane, by genetically fusing Mlc to the transmembrane protein LacY permease, was sufficient to derepress Mlc-controlled genes.[14] In contrast, targeting the LacI repressor to the inner face of the *E. coli* cytoplasmic membrane, by genetically fusing LacI to bacteriophage M13 coat protein, did not impede LacI-mediated repression of a tac operator-promoter located either on a plasmid or integrated into the chromosome.[15] As proposed by Gorke *et al.*, it is possible that these seemingly contradictory observations derive from distinct mechanism by which tethering regulates the activity of transcriptional regulators or their interactions with DNA. However, it remains unknown whether other regulators commonly used in synthetic biology may be amenable to spatially regulated control. Moreover, how or whether *conditional* spatial sequestration of transcriptional regulators may be engineered has not been explored.

Here we describe a platform for investigating and implementing conditional spatial sequestration of transcriptional regulators. We describe a mechanism for conditional tethering, in which expression of an exogenous (non-native) protease mediates proteolytic release of engineered transcriptional regulators from the inner face of the *E. coli* cytoplasmic membrane. We also demonstrate that this protease-alleviated spatial sequestration (PASS) mechanism can robustly mediate either conditional transcriptional activation or conditional transcriptional repression. Thus, this work both provides new insights into a biologically important facet of microbial gene regulation and establishes a new class of conditional regulation for the microbial synthetic biology toolbox.

RESULTS AND DISCUSSION

Protease-Alleviated Spatial Sequestration (PASS) Platform Design. Figure 1 summarizes the platform developed for evaluating the feasibility of the protease-alleviated spatial sequestration (PASS) concept. The proposed mechanism is as follows: (1) the transcriptional regulator is tethered to the inner face of the cytoplasmic membrane by genetically fusing the regulator to an engineered single-pass integral membrane protein, separated by a sequence that is labile to cleavage by an exogenous protease (hereafter, the PASS construct); (2) expression of the exogenous protease enables it to cleave the PASS construct, liberating the transcriptional regulator to diffuse into the cytoplasm and bind DNA. Note that "exogenous" is used herein to indicate that the DNA encoding the protease is not native to the *E. coli* host, not to indicate that the protease is extracellular. In our system, PASS constructs included an N-terminal ectodomain based upon the monomeric red fluorescent protein mCherry,[16] which was targeted for Sec-mediated transport to the periplasm *via* fusion to a MalE signal sequence (MalE_SS). The single-pass α-helical transmembrane domain was derived from the native *E. coli* ATP synthase subunit B, for which the structure is known.[17] The protease derived from tobacco etch virus (TEV) was selected

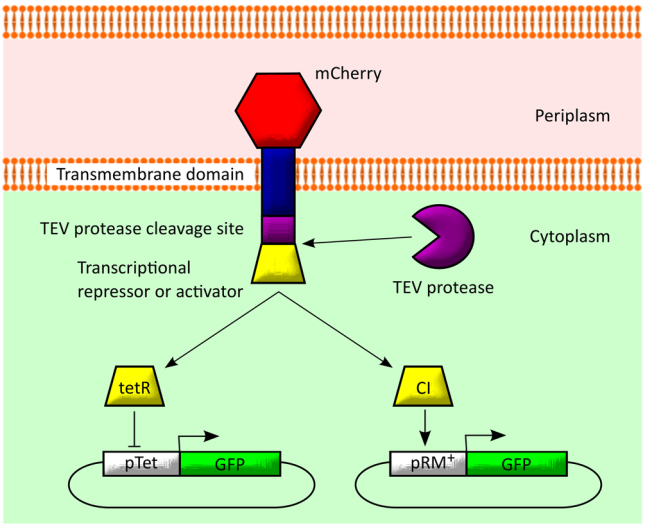

Figure 1. PASS concept and mechanism of action. The proposed mechanism is as follows: tethering a transcriptional regulator to the inner face of the cytoplasmic membrane prevents its ability to regulate target gene expression; protease-mediated cleavage of the PASS construct liberates the transcriptional regulator to repress or activate its cognate promoter sequence. In this study, recombinant PASS constructs included a periplasmic mCherry ectodomain, a transmembrane α-helix derived from *E. coli* ATP synthase subunit B, the cleavage sequence for tobacco etch virus protease (TEV), and either the tetR or λ CI transcriptional regulator domains. Released tetR represses the constitutive pTet promoter, and released CI activates the conditional pRM$^+$ promoter, to modulate output gene (GFP) expression.

based upon its high degree of sequence specificity,[18,19] and all PASS constructs included the canonical TEV substrate sequence, ENLYFQ/G, where the slash indicates the cleavage site. Notably, TEV has been harnessed for intracellular protein processing in *E. coli in vivo*, suggesting that off-target cleavage may not be problematic.[20]

To evaluate the potential for PASS-mediated repression as well as induction of transcription, we selected a model transcriptional repressor and a model transcriptional activator. To evaluate repression, PASS constructs included the tetracycline-regulated repressor (tetR) and were evaluated with reporter plasmids driving the expression of GFP from the constitutive, tetR-regulated promoter pTet (Figure 1).[21] To evaluate activation, PASS constructs included a constitutively active transcriptional activator based on the CI transcription factor from λ phage.[22] The λ CI protein activates the λ pRM promoter, and reporter constructs were developed based upon the pRM$^+$ variant of this promoter in which OR3 was deleted to prevent repression of pRM at high concentrations of CI.[23] Finally, 6xHis tags were appended to C-termini of transcription factors to facilitate biochemical analysis. PASS constructs were expressed in an IPTG-inducible fashion from the pLacIQ promoter, and TEV was expressed in an arabinose-inducible fashion from the pBAD promoter.

To initially assess the quantitative range of our reporter systems, plasmids encoding arabinose-inducible tetR and CI (pBAD-tetR and pBAD-CI) were cotransformed with their cognate reporter plasmids (pTet-GFP and pRM$^+$-GFP), induced, and evaluated by microplate-based fluorescence analysis (Figure 2). Both tetR and CI conferred significant repression or induction, respectively, of GFP expression,

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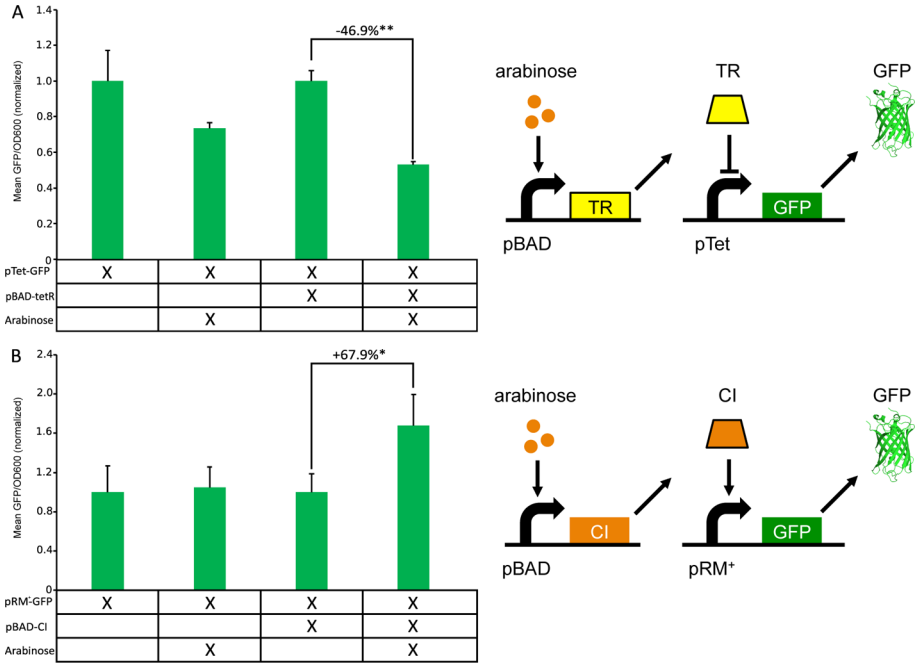

Figure 2. Regulation of reporter constructs by soluble repressor and activator. (A) Cells were transformed with the tetR-regulated reporter plasmid and tetR expression plasmid, as indicated, and induced with 1% (w/v) arabinose. (B) Cells were transformed with the CI-regulated reporter plasmid and CI expression plasmid, as indicated, and induced with 1% (w/v) arabinose. Samples undergoing exponential growth were analyzed, blanked, and normalized as described in Methods. Experiments were conducted in biological triplicate, and error bars indicate standard deviations. $*p < 10^{-5}$ and $**p < 10^{-10}$, as calculated for a two-tailed paired Student's *t* test. Abbreviations: TR, tetR.

confirming operability of the reporter system. Arabinose did confer some tetR-independent reduction in GFP for pTet-GFP containing cells, but this reduction was substantially less than the tetR-mediated reduction in GFP expression. Nonetheless, subsequent microplate analyses were controlled and normalized to account for this effect (see Methods). These soluble transcription factor controls also established benchmarks for interpreting the relative magnitude of subsequent PASS-mediated changes in reporter gene expression.

Inducible PASS Construct Expression and Cleavage. To evaluate expression of PASS constructs based upon membrane-bound tetR (mTR) or CI (mCI), fluorescence of the mCherry domain was assessed by microplate reader assays following induction with IPTG (Figure 3A). Most importantly, these data indicated that PASS constructs were expressed in a sufficiently stable fashion to enable mCherry folding and maturation. In addition, while IPTG treatment enhanced expression of both constructs, some expression was also evident in the absence of IPTG, presumably due to leaky expression from pLacIQ on high copy number plasmids.[24] To evaluate whether PASS constructs were proteolytically processed in accordance with the proposed mechanism, we analyzed this process in greater detail focusing on the mTR constructs. To this end, mTR constructs were coexpressed with TEV (driven by pBAD-TEV) and evaluated by Western blot (Figure 3B–D). N- and C-terminal fragments were detected *via* anti-mCherry and anti-6xHis antibodies, respectively. The full-length 55.6 kDa mTR construct was observed for all strains including the pLacIQ-mTR construct, in a manner that increased with IPTG but was not dependent on this inducer, which is consistent with the microplate assay analysis (Figure 3A). Arabinose-mediated expression of TEV dramatically increased the prevalence of the expected N- and C-terminal cleavage fragments of 31.4 and 24.2 kDa, respectively, while decreasing prevalence of the full-length mTR band. The cleavage products were also present at low levels in the absence of arabinose, potentially due to slightly leaky expression of TEV protease.

Several unexpected bands were also observed. Expression of full-length mTR corresponded with appearance of a band at ∼40 kDa, which was observed in both anti-mCherry and anti-6xHis blots. Because overexpression of MalE_SS-tagged mCherry leads to aggregation in the cytoplasm in a manner that blocks secretion,[25] we hypothesized that under high levels of expression, some mTR aggregates in either the cytoplasm or after transport to the cytoplasmic membrane. If the aggregated mTR were partially degraded near the N-terminus, this would reduce construct size without ablating binding by either the anti-His6X antibody or the anti-mCherry antibody (which binds within amino acids 84–237 of mCherry; personal communication with Abcam, June 26, 2014). Notably, this proposed proteolysis did not result in liberation of substantial quantities of soluble tetR (Figure 3B), such that this effect is unlikely to impede evaluation of the PASS mechanism. The aggregate also appeared partially labile to TEV-mediated cleavage, yielding an mCherry+ band of ∼15–20 kDa in the presence of TEV. As suggested by Figure 3A, substantial mTR was expressed in the absence of IPTG, and mTR expression increased with IPTG; in either case, induction of TEV with arabinose resulted in liberation of soluble tetR (Figure 3C,D). Altogether, these analyses indicated that mTR was generally expressed and processed as per the proposed PASS mechanism. Moreover, our conclusions pertaining to the proposed aggregation and proteolysis of mCherry are not specific to PASS constructs incorporating tetR, and thus this analysis likely applies generally to PASS constructs in which the periplasmic domain is based upon mCherry (including mCI).

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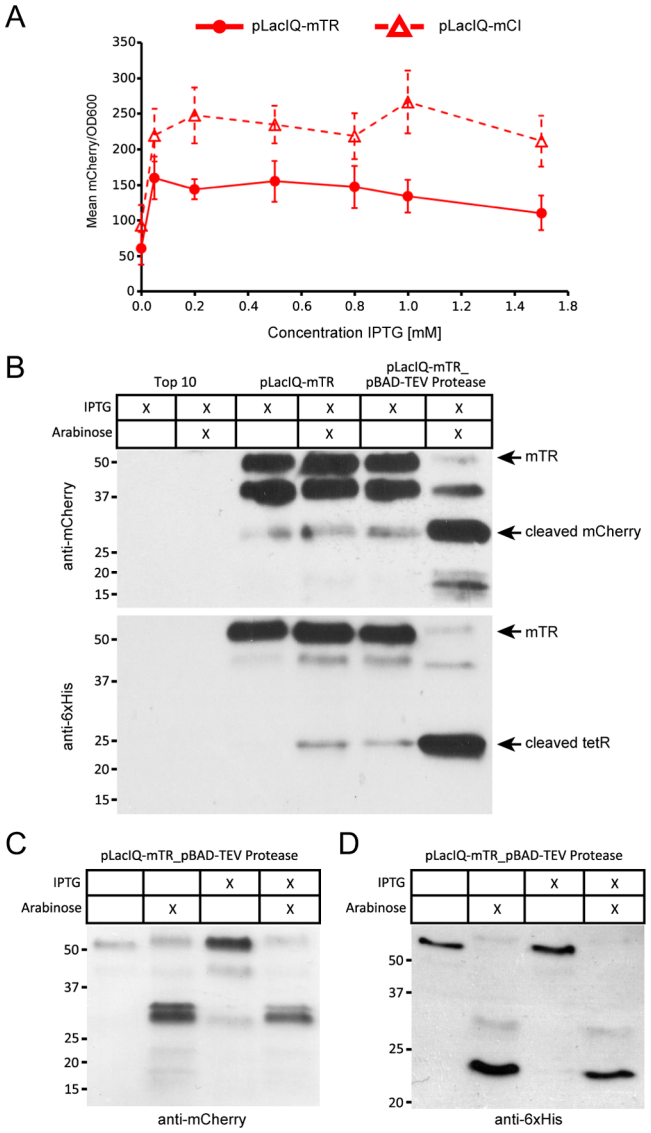

Figure 3. Expression and proteolytic processing of PASS constructs. (A) Cells were transformed as indicated and induced with varying concentrations of IPTG. Fluorescence was quantified as in Figure 2. Experiments were conducted in biological triplicate, and error bars indicate standard deviations. (B–D) Cells were transformed and induced, as indicated, with 1 mM IPTG and/or 1% (w/v) arabinose, and lysates were analyzed by N-terminal (mCherry) or C-terminal (6xHis) labeling. Protein standards are given in kilodaltons (kD).

Subcellular Localization of PASS Constructs. To determine whether PASS constructs were successfully integrated into the cytoplasmic membrane, we next visualized cells by fluorescent microscopy. Cells coexpressing cytoplasmic GFP and mTR exhibited a mCherry+ halo surrounding a GFP+ core (Figure 4A). Thus, the mCherry domain was successful secreted to the periplasm and refolded to reconstitute fluorescence. To determine whether this mCherry was associated with the cytoplasmic membrane (rather than simply secreted into the periplasm), we next generated spheroplasts by lysing the outer membrane to enable diffusion of soluble periplasmic species away from the cells.[26] For cells expressing either mTR and mCI, membrane-associated mCherry was retained upon conversion of intact cells (Figure 4B) to spheroplasts, which exhibited characteristic rounded morphology (Figure 4C). Together with the Western blot analysis, these data indicate that PASS constructs were efficiently integrated into the cytoplasmic membrane as designed, where they preferentially accumulated.

PASS-Regulated Repression of Gene Expression. Having established that the PASS mechanism functions as designed, we next investigated whether mTR repressed reporter gene expression from pTet in a manner that depended on TEV expression (Figure 5). These experiments included a control series in which mCI was expressed in place of mTR, in order to control for potential nonspecific impacts of PASS construct expression on GFP expression from the pTet-GFP reporter. Induction of TEV expression by arabinose led to a significant decrease in GFP expression, and the magnitude of this decrease was comparable to that mediated by soluble tetR (Figure 2). Repression was not enhanced by IPTG, although such treatment did increase expression of mTR (Figure 3). Thus, together, these data indicate that the low level of mTR expressed in the absence of IPTG provided sufficient tetR to maximally regulate the pTet promoter, and that TEV-mediated processing of mTR was efficient. Notably, no such IPTG- or arabinose-induced changes in GFP expression were observed for control cells expressing mCI in place of mTR. Moreover, these data indicate that subcellular sequestration of tetR to the inner surface of the cytoplasmic membrane *via* the PASS mechanism can limit this transcription factor's ability to suppress its cognate promoter. Notably, sequestration prevented repression of pTet encoded on high copy number plasmids; whether this holds true for chromosomally integrated pTet remains to be determined. Our observations also differ from those in which LacI was tethered in a similar fashion.[15] One potential explanation is that tethering tetR impacts its folding or DNA binding in a manner that is distinct from the mechanism by which tethering LacI impacts its regulatory capacity. Overall, tetR-based PASS constructs were functional and robust. One unexpected observation was that IPTG-induced expression of mTR (without arabinose) also conferred a reduction in reporter GFP expression, at least at the population-averaged level. Since IPTG-mediated induction of mTR did not alter overall bacterial growth compared to control cells in which IPTG induced expression of mCI (not shown), a simple growth effect is unlikely to entirely explain these results. Thus, we next investigated these phenomena at the single cell level in order to elucidate the mechanism by which mTR regulated reporter gene expression.

To evaluate whether individual cells within the population exhibited differential PASS-mediated conditional gene expression, we leveraged the fact that mTR constructs are fluorescent to analyze PASS function by flow cytometry. Consistent with Western blot and microplate analyses, TEV-mediated cleavage of mTR drove repression of pTet-GFP, even at uninduced levels of mTR expression for which mCherry expression was indistinguishable from background (Figure 6A). Within the population of mCherry+ cells, arabinose-induced expression of TEV drove a substantial suppression of pTet-GFP, for both basal and induced levels of mTR coexpression (Figure 6B), and the magnitude of this suppression surpassed that observed in population-averaged quantification by microplate assays (Figure 5). Thus, while IPTG-mediated induction of mTR may moderately suppress GFP expression in cells expressing pTet-GFP, our single cell analysis revealed that this effect was far less important than was the TEV-mediated release of tetR to suppress pTet *via* the proposed PASS mechanism (even in

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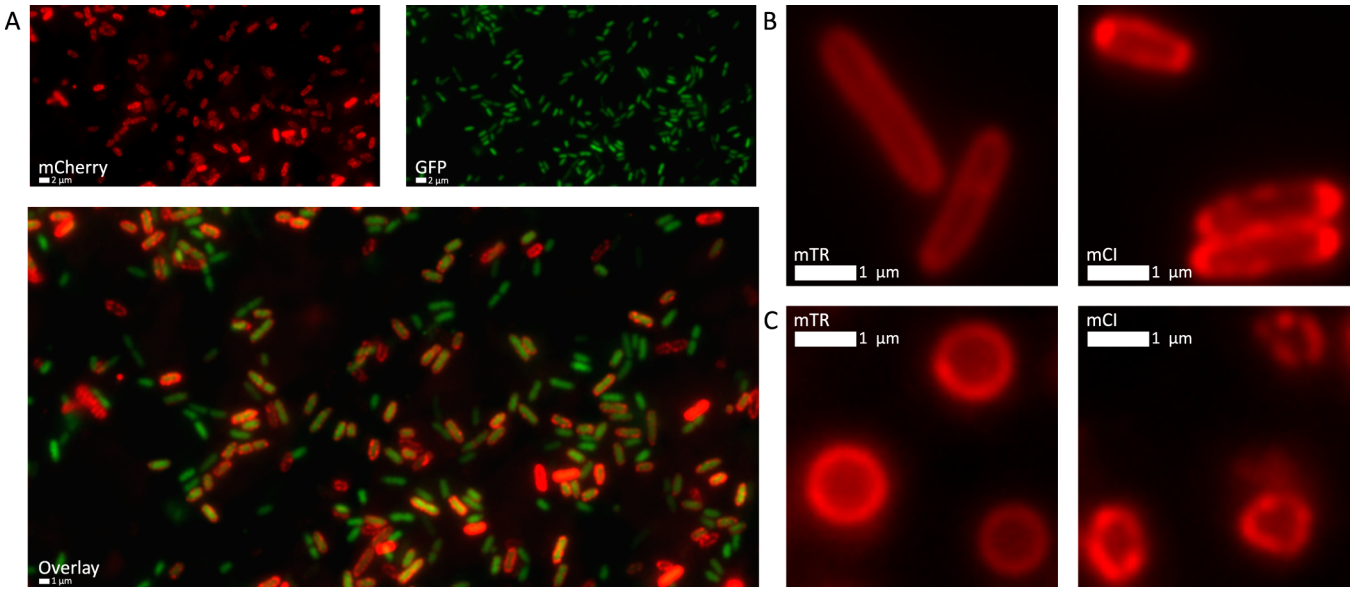

Figure 4. PASS construct localization at the cytoplasmic membrane. (A) Micrographs of cells transformed with pTet-GFP, pLacIQ-mTR, and pBAD-TEV Protease treated with 1 mM IPTG. mCherry fluorescence is localized to the periplasmic area while GFP fluorescence is diffuse throughout the cytoplasm. (B) Cells transformed with pLacIQ-mTR or pLacIQ-mCI and induced with 1 mM IPTG; shown in mCherry channel. (C) Spherolasts generated from cells in panel (B).

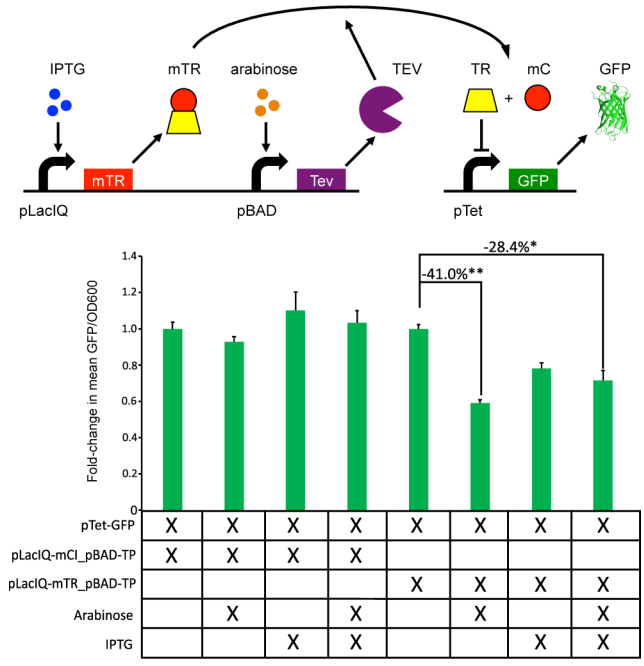

Figure 5. PASS-regulated gene repression by tetR. Cells were transformed and induced as indicated, and fold-change was quantified as in Methods. Experiments were conducted in biological triplicate, and error bars indicate standard deviations. $*p < 10^{-10}$ and $**p < 10^{-13}$, as calculated for a two-tailed paired Student's t test. Abbreviations: mC, membrane-bound mCherry (mTR cleavage product); TR, tetR.

mCherry+ cells). Although it is not clear why only a subset of cells expressed detectable levels of mCherry (as part of mTR), the PASS mechanism appeared to function robustly in this population. In addition, the arabinose (TEV)-responsive population was generally mCherry− in the absence of IPTG and mCherry+ in the presence of IPTG. Thus, there may exist a subset of cells or cell states in which the PASS mechanism functions most robustly, and we discuss opportunities for enhancing PASS performance below (see Conclusions).

PASS-Regulated Induction of Gene Expression. Having established that the mTR PASS system mediated conditional repression of gene expression, we next investigated whether the mCI PASS system mediates conditional activation of gene expression. In general, the results mirrored those observed with mTR, and these experiments similarly included a control series in which mTR was expressed in place of mCI. Induction of TEV expression by arabinose led to a significant increase in GFP expression from pRM$^+$ (Figure 7), and the magnitude of this increase was comparable to that mediated by soluble CI (Figure 2). TEV-mediated induction of gene expression did not require induction of mCI expression by IPTG, suggesting that leaky expression of mCI was sufficient to maximally activate the reporter in an arabinose (and thus TEV)-dependent manner. Moreover, these data indicate that subcellular sequestration of CI to the inner surface of the cytoplasmic membrane *via* the PASS mechanism can limit this transcription factor's ability to activate its cognate promoter. Single cell analysis by flow cytometry again indicated that only a subset of cells expressed mCI at levels detectable as mCherry+ (Figure 8A), and significant TEV-mediated induction of GFP was observed in mCherry+ cells (Figure 8B). To facilitate comparison, data in Figures 6 and 8 were collected with the same flow cytometer settings. The moderate induction of pRM$^+$-GFP by CI (compared to basal expression of GFP from pTet-GFP) is consistent with prior reports[23] and Figure 2, such that although the magnitude of mCI-mediated conditional gene regulation was less than that observed with mTR, this limitation likely reflects upon the dynamic range accessible to the CI system rather than the robustness of the PASS mechanism. Overall, these data collectively suggest that the PASS mechanism for conditional gene regulation may be applicable to a range of transcription factor platforms and mechanisms for achieving gene regulation.

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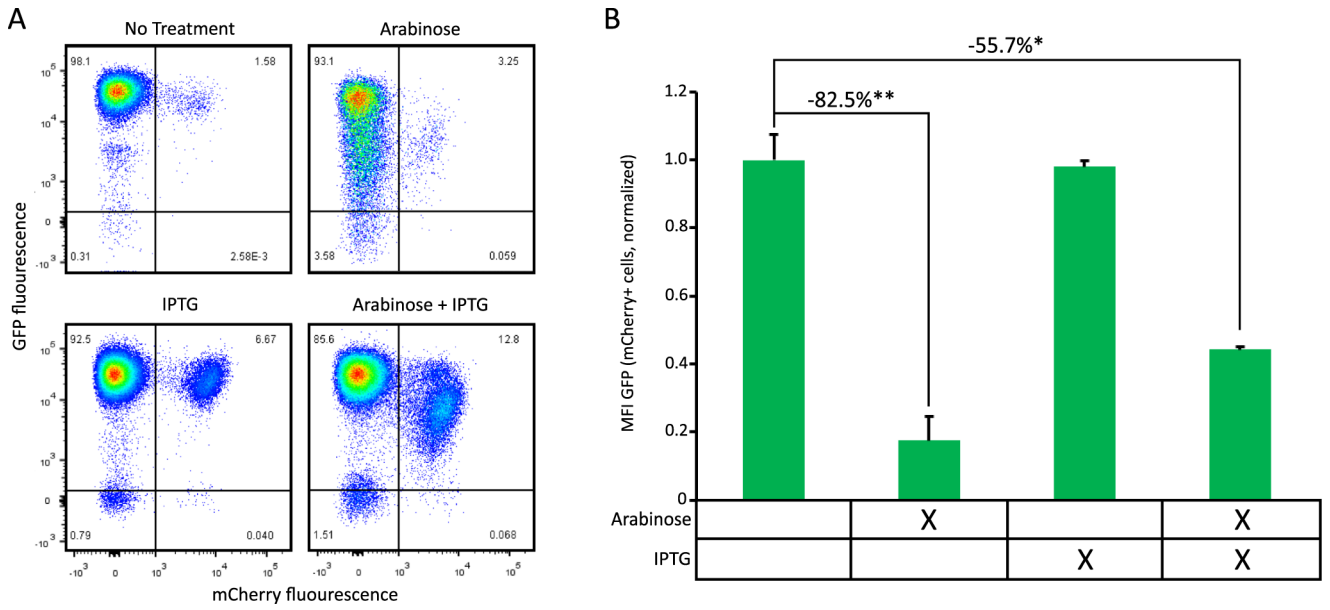

Figure 6. PASS-regulated gene repression in individual cells. (A) Cells transformed with pTet-GFP, pLacIQ-mTR, and pBAD-TEV were induced as indicated and analyzed by flow cytometry. (B) Mean fluorescence intensity (MFI) in GFP for mCherry+ cells from panel (A). Experiments were conducted in biological triplicate, and error bars indicate standard deviations. Normalized MFI GFP values were determined by calculating MFI GFP for mCherry+ cells in the test sample, calculating MFI GFP for mCherry+ cells in the uninduced sample, and then dividing the former by the latter. *$p < 0.01$ and **$p < 0.005$, as calculated for a two-tailed paired Student's t test. Additional flow cytometry plots for control cases are included in Figure S2.

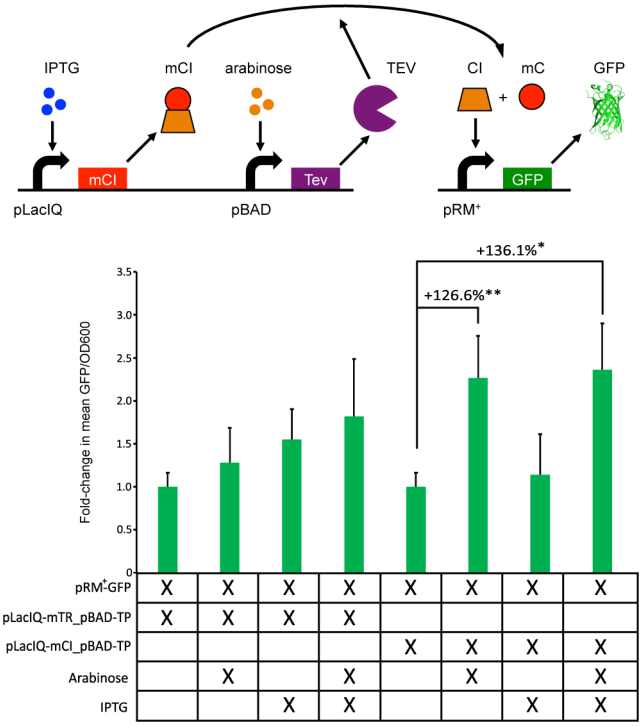

Figure 7. PASS-regulated gene repression by λ CI. Cells were transformed and induced as indicated, and fold-change was quantified as in Methods. Experiments were conducted in biological triplicate, and error bars indicate standard deviations. *$p < 10^{-3}$ and **$p < 10^{-5}$, as calculated for a two-tailed paired Student's t test. Abbreviations: mC, membrane-bound mCherry (mCI cleavage product).

CONCLUSIONS

The central objective of this study was to investigate a fundamental question in microbial gene regulation: is the engineered conditional sequestration of a transcriptional factor to the inner face of the cytoplasmic membrane a viable strategy for regulating gene expression in *E. coli*? We answered this query in the affirmative by establishing the feasibility of the PASS mechanism, and our results provide novel insights into microbial gene regulation and suggest strategies for harnessing this mechanism for biotechnology.

Given the feasibility of the PASS mechanism in our system, it is worth considering the conditions under which such regulation is possible. In this study, sequestration of either a repressor (tetR) or an activator (λ CI) precluded regulation of cognate promoters encoded on high copy number plasmids. Thus, this mechanism might be extensible to a range of regulator-promoter platforms, although the LacI system might represent an exception to this trend,[15] as discussed above. Multicopy plasmids with pUC19-derived origins, such as those on which reporters were encoded in this study, have been observed to cluster in subcellular foci,[27] which could help explain why sequestration was a robust regulator of transcription factor activity in our investigation. However, reporter plasmids used to evaluate sequestration of LacI used the same pUC19-derived origin (pMB1) and demonstrated no inhibition of repression upon tethering LacI to the cytoplasmic membrane,[15] so such plasmid clustering is seemingly not sufficient to mediate the effect of spatial sequestration of transcription factors. Subcellular spatial localization of chromosomal loci is highly regulated and dynamic,[28] and whether the PASS strategy can be extended to regulation of chromosomal promoters, and potentially promoters at different chromosomal locations, requires further investigation. Thus, PASS also provides a novel experimental tool for investigating gene regulation in the context of subcellular spatial organization and dynamics.

While this study focused on establishing the feasibility of the PASS mechanism, there now exist multiple opportunities for

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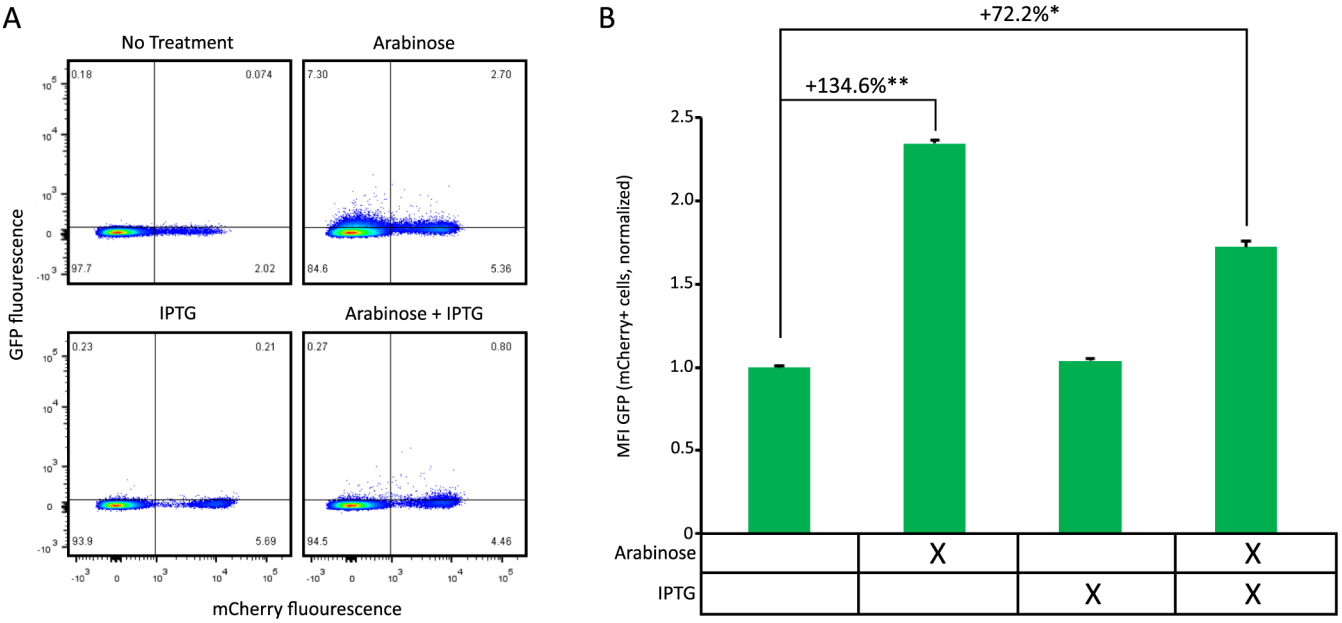

Figure 8. PASS-regulated gene induction in individual cells. (A) Cells transformed with pRM^{+}-GFP, pLacIQ-mCI, and pBAD-TEV were induced as indicated and analyzed by flow cytometry. (B) Mean fluorescence intensity (MFI) in GFP for mCherry+ cells from panel (A). Experiments were conducted in biological triplicate, and error bars indicate standard deviations. Normalized MFI GFP values were determined by calculating MFI GFP for mCherry+ cells in the test sample, calculating MFI GFP for mCherry+ cells in the uninduced sample, and then dividing the former by the latter. *$p < 10^{-2}$ and **$p < 10^{-4}$, as calculated for a two-tailed paired Student's t test. Additional flow cytometry plots for control cases are included in Figure S2.

optimizing PASS performance and leveraging this mechanism for synthetic biology applications. For example, use of lower copy number reporter plasmids would likely improve fold-change in output gene expression upon protease expression, particularly for reporter plasmids regulated by sequestered repressors (*e.g.*, pTet-GFP). Only a subset of cells expressed sufficient PASS constructs to appear mCherry+ by flow cytometry, and it is possible that PASS expression may be more uniform if these constructs were expressed at a lower level (*e.g.*, by using a less efficient ribosome binding site). Although our characterization experiments used inducible expression of TEV to "activate" the PASS construct, regulation of PASS could be made translation-independent in order to achieve the same rapid and robust responses exhibited by the natural systems discussed in the introduction (*e.g.*, the σ^{E} RIP and MalT systems), which inspired this investigation. To illustrate, we consider an application in which PASS is harnessed for biosensing. The TEV protease may be genetically split into fragments that individually lack catalytic activity, and catalytic activity may be reconstituted by bringing these fragments into proximity.[29] By fusing split-TEV fragments to protein domains that associate only in the presence of a small molecule,[30] protease activity is reconstituted in response to changes in cell state or environment. If a cell were engineered to constitutively express both these split-TEV biosensors and PASS constructs, then introduction of the small molecule analyte would trigger protease reconstitution, and these proteases could immediately act upon the large pool of presynthesized PASS constructs. Although such examples require direct experimental investigation, such a PASS-based mechanism could enable biosensors with higher sensitivity and rapid responses due to the intrinsic catalytic signal amplification within this proposed scheme. Altogether, PASS presents a new strategy for engineering microbial gene regulation to achieve the desirable performance characteristics exhibited by natural mechanisms while also being amenable to modular protein and gene circuit engineering.

METHODS

Media, Growth Conditions, and Strains. Cells were grown in Lysogeny broth (LB) Lennox formulation (10 g/L of tryptone, 5 g/L of yeast extract, 5 g/L of NaCl) for preparatory steps, and in supplemented M9 (M9 minimal medium with 0.4% glycerol, 0.2% casamino acids, and 1 mM thiamine hydrochloride) for characterization steps. All experiments were performed at 37 °C. Singly transformed cultures included 50 μg/mL kanamycin or 34 μg/mL chloramphenicol; doubly transformed cultures included 37.5 μg/mL kanamycin and 17 μg/mL chloramphenicol. All experiments were performed in TOP10 competent cells (Life Technologies), which have genotype: F-mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 Δ lacX74 recA1 araD139 Δ(araleu)7697 galU galK rpsL (StrR) endA1 nupG.

DNA Constructs. Primers were purchased from Life Technologies (Carlsbad, CA) and IDT (Coralville, IA), and *E. coli*-optimized GeneArt Strings for mCherry_TM, TM_PS_CI, and TM_TEV Protease were ordered from Life Technologies. All other coding sequences were obtained from the Spring 2010 Registry of Standard Biological Parts Distribution.[31] A complete list of DNA constructs as well as plasmids and primers used for cloning is included in Supporting Information.

Microplate Fluorescence Assays. Colonies were grown overnight in supplemented M9 medium with appropriate antibiotics and shaking, diluted to an OD600 of 0.05 in 3 mL of prewarmed M9 medium, and after reaching an OD600 between 0.4 and 0.6, cultures were again diluted to an OD600 of 0.05 in 2 mL of prewarmed M9 medium containing appropriate inducers and grown for 30−60 min. Three 180 μL replicates

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per culture were then transferred to black-walled clear-bottom 96-well plates (Corning) and placed in a Synergy H1MFD multimode microplate reader (BioTek). OD600, mCherry fluorescence, and GFP fluorescence were measured every 15 min for ∼10 h while shaking at 37 °C. Monochrometer excitation/emission settings were 585/615 nm for mCherry and 485/515 nm for GFP. In order to distinguish GFP-mediated fluorescence from autofluorescence, the fluorescence/OD600 value calculated for untransformed TOP10 cells was subtracted from each sample to generate "blanked" measures of GFP fluorescence/OD600, which we termed, "GFP/OD600". To calculate the mean value of (GFP fluorescence intensity per OD600) for each sample (here, Mean GFP/OD600), four consecutive time points from late exponential phase were selected for each sample (Figure S1). Late exponential phase was analyzed in order to ensure that cells were at steady state, rather than exhibiting transient responses to the inducer; all time points analyzed were at least 3 h after inducer addition. For each case analyzed, Mean GFP/OD600 was averaged over four time points for each of three biological replicates. We observed no pronounced construct-specific impacts on growth rate (not shown). To correct for nonspecific effects of inducers on reporter gene expression (see Figure 2), for each genetic configuration analyzed in Figures 5 and 7, fold-change in Mean GFP/OD600 upon inducer addition was normalized to the fold-change in Mean GFP/OD600 elicited by adding the same inducer(s) to a strain harboring only the corresponding reporter plasmid (pTet-GFP or pRM$^+$-GFP). Thus, by definition, fold-change for the inducer-free case for each genetic configuration was set to unity.

Spheroplast Production. Whole cells were converted to spheroplasts based upon an established protocol.[26] Briefly, cells were grown and induced as described for microplate assays, and samples were collected after 6 h of growth in microplate format. One milliliter of induced cell culture was centrifuged at 10000*g* for 4 min The pellet was resuspended in 100 μL of 30 mM Tris hydrochloride buffer (Tris-HCl, pH 8.0) containing 0.1 mg/mL of chloramphenicol and 3 mM NaN_3. Cells were again pelleted, the supernatant was removed, and the pellet was suspended in 100 μL of spheroplasting buffer (20% sucrose, 30 mM Tris-HCl (pH 8.0), 0.1 mg/mL chloramphenicol, 3 mM NaN_3). Ten microliters of a 1 mg/mL lysozyme solution (freshly dissolved in 0.1 M EDTA, pH 8.0) were added to each sample and incubated for 30 min at 0 °C. Spheroplasts were separated from released periplasmic contents by centrifugation at 10000*g* for 4 min.

Fluorescence Microscopy. Agarose pads were prepared by adding 2% low-melt agarose (Lonza) to minimal M9 medium and heating until melted. Thirty μL of agarose solution were then added onto a depression slide and covered with another flat slide (Fisher Scientific). Following pad solidification (∼10 min), 5 μL of cell culture were added to the pad, covered with a coverslip, and sealed with clear fingernail polish. Slides were viewed on an inverted fluorescent Leica DM-IL LED microscope with a Leica HCX PL APO 100×/1.40 PH CS oil-immersion objective lens and high-resolution cooled Q-imaging CCD. Images in Figure 4A were sharpened using the DeconvolutionJ plugin for ImageJ to reduce optical distortion.[32]

Western Blots. Colonies were grown overnight in 5 mL culture tubes in supplemented M9 medium with appropriate antibiotics, diluted to an OD600 of 0.05 in prewarmed M9 medium, and after reaching an OD600 between 0.4 and 0.6, cultures were again diluted to an OD600 of 0.05 in prewarmed M9 medium containing appropriate inducers. Samples were collected after reaching an OD600 of at least 0.1, diluted to an OD600 of 0.1 in M9 medium, and then combined with 2× Laemmli buffer. Samples were boiled at 95 °C for 5 min. Thirty microliters per sample were loaded and run in precast 12% (Figure 3D) or 4−15% gradient polyacrylamide gels (Bio-Rad). Gels were transferred to PVDF membranes (Bio-Rad) for 2 h at 100 V. Antibodies used for Western blot analyses were anti-mCherry monocolonal (Abcam), anti-His6X polyclonal (Abcam), antirabbit HRP-conjugated secondary (Life Technologies), and antimouse HRP-conjugated secondary (Cell Signaling). Transferred membranes were blocked in blocking buffer (5% milk in TBST: 50 mm Tris, 150 mM NaCl, 0.1% Tween-20) for 1 h followed by primary antibody labeling in blocking buffer at 4 °C overnight. Membranes were washed 3× with TBST and stained with secondary antibody in blocking buffer for 1 h at 4 °C. Membranes were washed 3× with TBST, treated with ECL solution (Bio-Rad), and then exposed to films (GE).

Flow Cytometry. Cells were grown and induced as described for microplate assays, and samples were collected after 6 h of growth in microplate format. Samples were analyzed on an LSR II (BD), and mean fluorescent intensity (MFI) was calculated using FlowJo software (Treestar). A minimum of 4000 individual cells (typically out of ∼20 000 events) was analyzed per sample.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplemental figures, descriptions and GenBank sequence files for all plasmids, and sequences for all primers and DNA blocks synthesized for this investigation. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

mC, membrane-bound mCherry (cleavage product of PASS constructs); mCI, PASS construct: mCherry-TM-PCS-CI (from N- to C-terminus); MFI, mean fluorescence intensity; mTR, PASS construct: mCherry-TM-PCS-tetR (from N- to C-terminus); PASS, protease-alleviated spatial sequestration; PCS, protease cleavage sequence; TEV, tobacco etch virus protease; TM, transmembrane domain; TR, tetR

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