Programmable biomolecular switches for rewiring flux in *Escherichia coli*

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Synthetic biology aims to develop programmable tools to perform complex functions such as redistributing metabolic flux in industrial microorganisms. However, development of protein-level circuits is limited by availability of designable, orthogonal, and composable tools. Here, with the aid of engineered viral proteases and proteolytic signals, we build two sets of controllable protein units, which can be rationally configured to three tools. Using a protease-based dynamic regulation circuit to fine-tune metabolic flow, we achieve 12.63 g L\(^{-1}\) shikimate titer in minimal medium without inducer. In addition, the carbon catabolite repression is alleviated by protease-based inverter-mediated flux redistribution under multiple carbon sources. By coordinating reaction rate using a protease-based oscillator in *E. coli*, we achieve d-xylonate productivity of 7.12 g L\(^{-1}\) h\(^{-1}\) with a titer of 199.44 g L\(^{-1}\). These results highlight the applicability of programmable protein switches to metabolic engineering for valuable chemicals production.
ENGINEERING MICROBIAL CELL FACTORY TO PRODUCE VALUABLE CHEMICALS FROM RENEWABLE FEEDSTOCKS PLAYS AN ESSENTIAL ROLE TO IMPLEMENT SUSTAINABILITY. TO MAXIMIZE PRODUCT TITER, YIELD, AND PRODUCTIVITY, METABOLIC FLUX NEEDS TO BE FINELY PROGRAMMED WITHOUT DISRUPTING CELLULAR HOMEOSTASIS. CURRENT FLUX Rewiring Technologies Have Centered on the Transcriptional-Level Regulation Because of the Ease and Success of Sophisticated Engineering of Metabolite-Responsive Transcriptional Factors. Despite Unprecedented Achievements Making by Transcriptional Regulation, the Associated Long Response Time Can Lead to Faulted Genetic Circuits and Ultimately Fail to Control Metabolic Flux. Recently, Multilayer Regulation Involving Both Transcriptional- and Protein-Level Reprogramming Is Reported. Protein-Level Regulation in Those Systems Relies on the Modification of Target Proteins with Degradation Tags or Degrons. However, These Systems Suffer from a Number of Issues, Including Tunability and Orthogonality, and the Lack of Protein–Protein Interaction Regulators Have Limited the Ability to Design and Engineer Fast-Response Biomolecular Switches That Can Rapidly Reprogram Metabolic Flux and Persistently Maintain Cellular Homeostasis.

In This Study, Viral Proteases That Specifically Recognize and Cleave Short Cognate Target Sites Are Applied in Combination with Proteolytic Signals to Control Protein Stability and Residence Time in Escherichia coli. To Unravel the Design Principles Underlying Protease-Based Metabolic Switches, We Have Developed a Synthetic Biology Toolbox, Including Protease-Based Dynamic Regulation Circuit (pbDRC), Protease-Based Inverter (pbI), and Protease-Based Oscillator (pbO), to Achieve Fast-Response and Tunable Control of Metabolic Flux. These Rationally Designed Switches Exhibit Superior Applicability for the Regulation of Metabolic Flux and Improvement of Metabolite Production in the Industrial Workhorse E. coli.

RESULTS

Protease-Based Protein Regulatory Unit Construction. To Construct Bifunctional Switches That Enable E. coli to Accumulate or Degrade Target Proteins When Reprogramming Metabolic Flux, Two Basic Protein Regulatory Units (Including Off-Switch Unit and On-Switch Unit) Were Constructed. The Off-Switch Unit Consists of a Protease That Exposes the N-Degron on the Modified mCherry and Drives Its Degradation (Fig. 1a). The On-Switch Unit Contains a Protease That Specifically Removes the Degradation Tag of Modified Green Fluorescent Protein (GFP) and Protects It from Degradation (Fig. 1b). Depending on the Configuration and Orientation of the Proteolytic Tag and the Protease Cleavage Tag, This Protease-Based Switch Demonstrated Selective On–Off Control of Protein Degradation.

To Optimize the Dynamic Range of These Units, We Constructed a Library of Tobacco Etch Virus Protease (TEVp) Expression Variants with Different Strength of RBSs (Supplementary Fig. 1). The
proteolytic activity was screened, in which a 22.5-fold mCherry protein decrease in OFF-switch unit (Fig. 1c) and a 23.8-fold GFP increase in ON-switch unit was achieved (Fig. 1d). Kinetic analysis indicated that the processes of protein degradation and accumulation could be increased with higher inducer concentration, demonstrating that these basic protein regulatory units were tunable (Fig. 1e, f). Flow cytometry results demonstrated that both the protease-based ON/OFF-switch units exhibited good population homogeneity (Fig. 1g, h). To test system specificity, non-targeted mCherry protein was fused in both regulatory units. The expression of TEVp was able to downregulate the abundance of mVenus protein (yellow fluorescent protein (YFP)) by 27.2-fold in the OFF-switch unit and upregulated GFP abundance by 50.7-fold, which indicated that the ON/OFF-switch had excellent orthogonality in multi-protein processing (Supplementary Fig. 2).

In vivo kinetic results showed that the half-life of protein degradation in the OFF-switch unit ranged from 25 to 60 min, with a dead time of <10 min (Fig. 1i, j). Moreover, compared with the commonly used LacI-IPTG-inducible expression system, they also demonstrated perfect dose–response curve (Supplementary Fig. 3). Those results indicated that the basic protein regulatory units were highly specific and quick in response.

pbDRC design and characterization. Based on OFF-switch unit, the first tool we constructed was pbDRC (Fig. 2a). Both stationary phase promoter (SPP) and growth phase promoter (GPP) were contained in this circuit. Gene expression under the control of SPP will be repressed before the cell enters into stationary phase, whereas gene expression under the GPP will be repressed after the cell enters the stationary phase (Supplementary Fig. 4). To achieve tight control, a series of SPPs and GPPs were screened, among which four SPPs of different strengths (fic, bolA, S4, and S60) and three GPPs of different strengths (rrnB P1, rpsT P2, rpsJ) were selected (Supplementary Fig. 5).

Upon co-expression of SPP-driven TEVp and GPP-driven YFP, the resulting strains exhibited a variety of turning point in the reporter protein ranging from 7 to 10 h (Fig. 2b, c). Higher strength SPPs resulted in shorter switch time and lower reporter accumulation (Supplementary Fig. 6). To investigate the role of protease in pbDRC, constitutively expressed TEVp by promoter Ptet was co-expressed with either wild-type YFP or degradable YFP. Without degradation signal, the accumulation of wild-type YFP lasted for more than 24 h. However, a much shorter switch time (<4 h) in strains with degradable YFP demonstrated that TEVp-mediated proteolysis is indeed functional in the pbDRC (Fig. 2c). Furthermore, the growth curve confirmed that pbDRC had negligible pressure on cell growth (Fig. 2b). Thus, via a simple modification of promoters, YFP abundance could be dynamically controlled at different switch times by the pbDRC tool.

pbI design and characterization. Based on ON/OFF-switch unit, we attempted to construct a second biomolecular tool pbI. First, we tested the direct introduction of one protease to control the different fates of GFP and mCherry. Although the introduction of TEVp could tune the abundance of mCherry and GFP, we observed that a higher level of TEVp was beneficial for tuning GFP accumulation but was unable to effectively modulate mCherry degradation (Supplementary Fig. 7). This result suggested that there was a trade-off between protein accumulation and degradation, which could not be addressed by simply introducing one protease. Thus, to facilitate unit compatibility, the pbI tool should contain proteases with equivalent functions.
but different specificities. Our designed pbI consisted of two orthogonal proteases, TEVp and TVMVp (tobacco vein mottling virus protease), to tightly control the abundance of the reporters (GFP and mCherry) (Supplementary Fig. 8).

Two types of pbIs were constructed (Fig. 2d, e). The first pbI consisted of two orthogonal proteases to control reporter level in OFF-switch unit or ON-switch unit (Fig. 2d). The second pbI contained an additional layered OFF-switch unit connected with either ON-switch unit or OFF-switch unit (Fig. 2e and Supplementary Fig. 9). Inversion of GFP and mCherry abundance was observed in both types of pbIs, demonstrating pbIs exhibited good composability in protein circuit construction.

**pbO design and characterization.** We also constructed a third biomolecular tool pbO. Inspired by the classical example of the repressilator (Supplementary Fig. 10), we hypothesized that the reporter protein abundance could be periodically modulated with periodical protease input. Our oscillator ring structure consisted of three orthogonal—TEVp, TVMVp, and SuMMVp (sunflower mild mosaic virus protease) (Supplementary Fig. 10). Moreover, each was modified by fusing their N terminus with a degron and degraded by corresponding proteases. Reporter expression results indicated that modified proteases also exhibited excellent orthogonality and specificity (Supplementary Fig. 11). To further validate this hypothesis, either an individually expressed TEVp (Supplementary Fig. 12) or all three proteases co-expressed at the same strength (Fig. 3b) were incorporated into the pbO as a trigger. To analyze the effect of proteases cascade degradation on fluorescent protein accumulation, a control strain in which YFP was observed in both types of pbIs, demonstrating pbIs exhibited good composability in protein circuit construction.

**Dynamic flux regulation in shikimate production using pbDRC.** We started off by applying the pbDRC to control carbon flow. Shikimate (SHK), an important starting material in treating fluology microscopy results indicated that individual pbO cells could exhibit sustained oscillations compared with the control strains (Fig. 4). Collectively, those results indicated that pbO can be constructed by periodical protease input and fine-tuning is needed in the future to achieve a perfect oscillatory expression.

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**Fig. 3** The pbO design and characterization. **a** Control system without YFP degradation. **b** Three proteases trigger oscillator system. **c, d** Characterization of fluorescence curve of control strain (c) and pbO strain (d). Black trace was the mean value of discrete data points measured from six separate colonies. **e, f** Flow cytometry measurements of control strain (e) and pbO strain (f) at different time points. Source data of Fig. 3c and 3d are provided in Source Data file.
influenza, is traditionally produced by blocking metabolic flow from SHK to SHK-3-phosphate (S3P) (Fig. 5a). This eventually leads to the interruption of endogenous aromatic amino acid (AAA) synthesis and the sacrifice of cell growth. Exogenous AAAs can be supplemented to avoid defects in cell growth, which inevitably increases the manufacturing costs of SHK. An alternative solution is to decouple cell growth from SHK production. We therefore used an engineered E. coli S4 as a chassis, in which SHK kinases were deleted, phosphotransferase system was replaced by glucose facilitator from Zymomonas mobilis, and pathway enzymes encoding by tktA, aroG, aroB, and aroE were overexpressed (Fig. 5a and Table 1). This strain exhibited growth defects in New Brunswick Scientific (NBS) minimal medium.

Next, we engineered SPP-controlled TEVp into E. coli S4 to regulate GPP-controlled aroK (SHK kinase I), which converts SHK to S3P in the metabolic pathway. Strains (DS1–DS12) containing different combinations of SPPs and GPPs were assayed in NBS minimal medium without the addition of AAA and inducers, among which strain DS7 with a combination of promoter rpsT P2 and bolA exhibited a SHK titer increase from undetectable to 2.14 g L$^{-1}$ without cell growth sacrifice (Fig. 5b and Supplementary Figs. 13, 14).

To show the relationship between target enzyme degradation and titer improvement, the SHK kinase activity of four different strains including S4, D13 (SHK kinase I was expressed under the control of GPP, P$_{rpST}$P2), D14 (SHK kinase I was constitutively expressed, but would be degraded by SPP-based induced TEVp), and DS7 (GPP-based repression of SHK kinase I combined with SPP-based induced degradation) were evaluated during the SHK production (Supplementary Fig. 14 and Table 1). As shown in Fig. 5d, SHK kinase could be degraded in the same manner as the fluorescent protein, given the fact that the SHK kinase activity of strain DS7 at 36 h was only 0.16-fold of that at 6 h. Moreover, we observed a direct negative correlation between SHK kinase activity and SHK titer (Fig. 5c).

To determine the applicability of pbDRC in bench-top bioreactors, the performance of strain DS7 in a 5 L fermenter was investigated. Compared with that of shaker flasks, a 5.9-fold higher SHK titer (12.63 g L$^{-1}$, yield of 0.19 g g$^{-1}$ glucose) was achieved (Fig. 5e). Moreover, to evaluate pbDRC as a universal tool, other seven E. coli variants including DH5α, JM109, W3110, ATCC 8739, B0013 (derived from E. coli K-12); BL21 (derived from E. coli B); and HB101 (a hybrid of the E. coli K-12 and B) were engineered at the same sites, according to strain DS7. As shown in Fig. 5f, all the pbDRC-containing strains achieved increased SHK titer ranging from 7.3- to 42.8-fold compared with that of the control strains, respectively. Furthermore, genomic integration of pbDRC was also attempted in E. coli MG1655 by replacing the wild-type aroK with the rpsT P2 promoter-driven cleavable aroK. All strains with SPP-controlled TEVp exhibited a 3.6- to 8.5-fold titer (0.79–1.88 g L$^{-1}$) without acetate accumulation in comparison with the control strain (Fig. 5g). Overall, pbDRC provided dynamic, robust, strain-independent regulation of metabolic flux flow at scales of industrial relevance.
**Fig. 5** Dynamic regulation for shikimate production. 

**a** Engineering shikimate biosynthetic pathway in chassis *E. coli* strain S4. Red cross indicated pathway blocking. Genes that are bold were overexpressed.

**b** Effect of different combinations of the pbDRC on titer and cell growth (72 h). + and − represent chassis S4 cultured in NBS medium with (+) or without (−) AAA supplement. Insert was orthogonality matrix for strains construction.

**c** Titer comparison of different strains in NBS medium without AAA supplement. Insert: arOK/arOL represents the genomic expression of shikimate kinase I/II; GPP-arOK represents the expression of shikimate kinase I under the control of GPP; SPP-tev represents a SPP-based expression of TEVp with a constitutive expression of shikimate kinase I.

**d** Evaluation of shikimate kinase I degradation in different strains during shikimate production.

**e** Cell growth (OD600) and shikimate production (SHK) of different strains in NBS medium without AAA supplement. Insert: arOK/aroL represents the genomic expression of shikimate kinase I/II; GPP-arOK represents the expression of shikimate kinase I under the control of GPP; SPP-tev represents a SPP-based expression of TEVp with a constitutive expression of shikimate kinase I.

**f** Evaluation of shikimate degradation in different strains during shikimate production.

**g** Genomic-scale dynamic pathway modulation of shikimate production (72 h). – represents strain without expressing TEVp. Values are shown as mean ± SD (n = 3).

**Supplementary Information**

aroBopt, DHQ synthase with first eight codons optimized; aroD, DHQ dehydratase; aroE, shikimate dehydrogenase; arOGfr, feedback-resistant mutants of DAHP synthase (D146N); arOK/arOL, shikimate kinase I/II; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; DHQ, 3-dehydroquinic acid; DHS, 3-dehydroshikimate; E4P, erythrose-4-phosphate; G3P, glyceraldehyde 3-phosphate; G6P, glucose 6-phosphate; PEP, phosphoenolpyruvate; PTS, phosphotransferase system; PP pathway, pentose phosphate pathway; PYR, pyruvate; SHK, shikimate; S3P, shikimate-3-phosphate; tktA, transketolase A; Zmglf, glucose facilitator from *Z. mobilis*. Source data of Fig. 5b–g are provided in Source Data file.
Flux direction regulation in D-xylonate production using pbI. The second application was to control the direction of carbon flux using pbI. D-xylolactone, one of the top 30 high-value chemicals, was currently produced with pure D-xylose, which greatly increased production costs17. Attempts at decreasing the production cost have focused on using low-cost substrates, such as cellulose hydrolysate18. However, the major obstacle in this process was carbon catabolite repression, which prolongs the uptake of D-xylose when enough glucose is present19 (Supplementary Fig. 15).

A promising strategy to address this issue is to block glucose utilization after the biomass reaches a certain density using pbI. To test this, a chassis of E. coli X3 was constructed by introducing cleavable phosphotransferase enzyme I (PTSI) in a D-xylose, D-xylolactone, and glucose catabolic defects strain X2 (Fig. 6a and Table 1). Then, a pbI, which contained TVMVP-cleavable D-xylose dehydrogenase, TEVP-cleavable TMVp, TEVP-cleavable PTSI, and TEVp, was co-expressed in strain X3 (Table 1 and Supplementary Fig. 14). The resulted strain X4 exhibited a growth defect phenotype in the ATC pre-added glucose-NBS minimal medium. This phenotype was consistent with a PTS defect strain X2, suggesting that the activity of phosphotransferase system can be artificially controlled by introducing pbI (Fig. 6b). Subsequently, we tested the effect of different induction time on strain growth and xylolactone production. Result of the induction time in strain X4 demonstrated that higher D-xylolactone production (4.25 g L⁻¹) could be achieved when induced after 6 h of incubation (OD₆₀₀ = 0.78) (Fig. 6c). On this basis, strain X4 exhibited comparable D-xylolactone titer under different sugar ratios in mixed sugar culture conditions. Notably, when the sugar ratio was controlled at 2 (glucose vs. xylose), a 1.79-fold titer increase (4.25 g L⁻¹) was achieved over that of the control strain (Fig. 6d). Those results revealed that pbI effectively rerouted the direction of metabolic flux, which has a great potential in D-xylolactone or other high-value compounds production from mix substrates.

Flux rate regulation in D-xylonate production using pbO. The third application is using pbO to tune carbon flux rate. Previous studies suggested that a higher D-xylolactone titer could be achieved at an early stage of culture by co-expression of xylose dehydrogenase and lactonase20. However, given the fact that lactonase-mediated hydrolysis of D-xylolactone to D-xylolactone acidifies the cytoplasm, thereby decreasing cell viability, which ultimately compromises D-xylonate productivity21, the current D-xylonate titer and productivity produced by engineered E. coli was less commercially competitive by individual expression of xylose dehydrogenase (39.2 g L⁻¹ with 1.09 g L⁻¹ h⁻¹)22 or simply by co-expressing xylose dehydrogenase and lactonase at a low level (108.2 g L⁻¹ with 1.8 g L⁻¹ h⁻¹)23. Therefore, increasing D-xylonate titer relied on the precise control of the cascade reaction rate.

We reasoned that periodically slowing down the downstream reaction rate of the cascade should improve xylolactone production without impairing cell viability (Fig. 7a). To validate this hypothesis, engineered strain X1 with D-xylolactone and D-xylose catabolic defects was chosen as chassis for pbO demonstration (Table 1). By co-expressing xylose dehydrogenase, TEVp-cleavable lactonase, and trigger plasmid pTet-(tvF)suumv-(suF)tev-(teF)tvmv, the resulting strain, XO, harboring pbO was constructed (Supplementary Fig. 14). As a result, a 2.02-fold titer (6.88 g L⁻¹), with a yield of 1.04 g L⁻¹ h⁻¹, was achieved in the strain XO, compared with that of the negative control strain XN (Table 1 and Supplementary Fig. 14), although they had similar biomass accumulation (Fig. 7b and Supplementary Fig. 16).

To investigate the mechanism underlying titer improvement, a pH reporter, pHluorin, from Aequorea victoria was expressed21. The Rₐ₅₉₄₇₅ values obtained from cells containing pHluorin protein could be used to quantify the intracellular pH (in vivo pH), because they exhibited a biphasic relationship to pH from 3 to 8 (Supplementary Fig. 17). In addition to the strain XN, a positive control strain (XP) with xylose dehydrogenase but without lactonase overexpression was constructed (Supplementary Fig. 14). As shown in Fig. 7c, compared with XP, strains co-expressed with xylose dehydrogenase and lactonase (XN and XO) exhibited a good D-xylolactone production capacity according to results from the extracellular pH (in vitro pH). Moreover, by introducing protein oscillator, intracellular pH (in vivo pH) homeostasis was achieved in strain XO, which further led to the observed increase in cell viability by more than two orders of magnitude in 36 h compared with that of the control strain XN (Fig. 7d and Supplementary Fig. 18). Lastly, culture conditions were optimized, in which a D-xylolactone titer of 13.11 g L⁻¹ was achieved using Terrific Broth (TB) medium at 37°C in shaker flasks (Supplementary Fig. 19). Under such conditions, results of engineered XO strain in a 5 L fed-batch culture demonstrated the highest D-xylolactone titer and productivity could reach 199.44 g L⁻¹ and 7.12 g L⁻¹ h⁻¹, respectively (Fig. 7e). In summary, pbO provide a advantageous tool for precisely controlling metabolic events in an engineered cell factory.

Discussion
In this study, we constructed two composable protein regulation units, which could be rationally applied in the design of three biomolecular toolkits—pbDRC, pbI, and pbO. Experimental results clearly demonstrated that these carbon flux regulation tools could perform sophisticated functions and exhibited good applicability in optimizing carbon flux under various scenarios of metabolic engineering practices.

The protease-based ON/OFF-switch exhibited good composability and extendibility in protein-level circuit construction. Compared with the transcriptional or posttranscriptional regulations reported in previous studies (the input relied on small-molecule inducers24,25 or nucleic acids26,27 and output depended on transcription factors or nucleic acid-interacting proteins), the input and output of protease-based circuits could be standardized to orthogonal proteases. Thus, by rationally configuring
engineered viral proteases and proteolytic signals, distinct ON/OFF switches could be created and connected to each other to yield predictable behavior.\(^{28,29}\). Recently, a series of protein circuits was constructed in mammalian cells by introducing leucine zipper motifs and split proteases.\(^{28,30}\). Different from those studies, an alternative approach for constructing tunable protein circuits in \(E. coli\) was provided in this work by controlling the input protease dosage. Moreover, the types of protein circuits were further expanded by developing three protease-based biomolecular toolkits.

On the principle that enzyme activity is associated with its abundance, these tools could be used for precisely tuning carbon flux, direction, and rate. Base-pairing interactions at the transcriptional level could offer numerous programmable technologies, but there is a common drawback of prolonged response times.\(^{4,6,30}\). Although proteases could respond rapidly to cleave and induce the degradation of target proteins,\(^{28,31}\), its orthogonality with the host makes it difficult to sense metabolite concentration changes or the state of the host for making corresponding dynamic adjustments. In this study, we demonstrated that an intelligent circuit, pbDRC, could be constructed by additional integration of transcription-level regulation on protein-level circuit. By rational combinations of GPPs and SPPs to trigger the production of proteases to obtain ON or OFF status of metabolic proteins, we provided a valuable alternative to previous QS-dependent dynamic regulation system.\(^{11}\). Moreover, due to the independence of homologous degradation machinery in \(E. coli\), our pbDRC platform exhibited general applicability across \(E. coli\) strains and demonstrated robust performance at scales of industrial relevance.

Compared with previously reported transcriptional-level inverters that relied on CRISPR\(^{32,33}\), zinc finger proteins, small RNAs,\(^{34}\), and antisense RNA,\(^{35}\), here, a pbI was constructed, which exhibited transcriptional-independent characteristics, i.e., protein functionality can be changed, even after the cognate mRNAs have been translated.\(^{36}\) This could be beneficial for metabolic engineering cases where product synthesis competes with biomass formation of common precursors.\(^{37}\). Furthermore, the rapid response kinetics involved in protein-level regulation enabled us to build inverters with broader dynamic ranges and precise time-switching functions than previously reported inverters.\(^{35}\).

The inherent nonlinearity of protease input and protein degradation output can offer oscillatory regulation with shorter periods than transcriptional-level regulation. Previously, a classic oscillator was developed using three orthogonal transcriptional regulators with a cycle time of 160 ± 40 min.\(^{38}\). In this study, with a shorter signal conduction chain, pbO achieved an oscillatory period closer to 90 min. Moreover, the robustness of our protein oscillator was improved by coordinating...
protease input, indicating that pbO display high levels of inter-connectedness. In practical applications involving metabolic flux rate tuning, this protein oscillator exhibited unique application potential. Compared with the direct overexpression of lactonase, periodically regulating lactonase reaction rates using protein oscillators could achieve a 1.84-fold higher D-xylonate titer and a 4-fold productivity greater than previous reports in engineered E. coli.\(^3\) We expected that by tuning the input dose of protease, the frequency and intensity of oscillators could be further optimized to address metabolic engineering problems, such as the modulation of metabolic pathways involved in toxic intermediate accumulation\(^7\) or accurate synthesis of target chemicals with a specific molecular weight range\(^37\).

Although protease-based posttranslational regulation offers many advantages on rapid response, composability, and modularity\(^28,39\), some limitations should also be taken into account when constructing protein circuits. The first one is the ATP cost associated with proteolysis. As the unfolding and translocation of protein substrate are energetically unfavorable, the total ATP cost of ClpP-mediated proteolysis can be relatively high. For example, about 600 ATPs will be consumed in the degradation of a model substrate titin\(^127\) domain\(^40\). This energy burden caused by introducing controllable proteolysis may affect both cell proliferation and genetic circuit output. Thus, it is necessary for decreasing intracellular resource occupancy by fine-tuning the output strength of artificial circuits in the design and implementation of protein circuits\(^39\). The second limitation is the queuing effect. When multiple proteins are designed to be degraded and competed for proteolytic resources, the ClpP machinery will be overloaded. As a result, the unexpected queuing coupling response can occur, which will decrease target protein degradation rate, affect the innate parameters of genetic parts and subsequently change the profile of circuits output\(^41\).

One promising strategy to overcome queuing effect is to exploit heterologous proteolysis machinery, such as Mesoplasma florum Lon protease complexes\(^12\).

Transcriptional regulation systems are frequently used in metabolic engineering, most commonly for the overexpression of pathway enzymes and for tight regulation of toxic products. In this study, three protease-based circuit systems were able to complement current transcriptional regulation systems and expand their versatility by harnessing reprogrammable protein regulation units. With this protease-based synthetic biology toolbox, protease-based circuits involving protein-level regulation are expected to advance metabolic engineering and synthetic biology.
Strains and plasmids. Strains used for the implementation of prokaryotic-based tools in this study are listed in Table 1. All constructs used for tools development were generated using ligatog cloning procedures from Takara Bio (Dalian, China) or one-step cloning kit procedure from Vazyme Biotech (Nanjing, China). A list of all genetic components and plasmids reported in this manuscript are included in Supplementary Data 1, Supplementary Fig. 20, and Supplementary Tables 2-7. DNA sequences of those plasmids can be found in Supplementary Data 1.

For SHK production, E. coli MG1655 was used for chassis strain S4 construction. The gene aroE was genomically replaced by a tktA expression cassette under the control of promoter rpsT P2 in chassis S4. Pathway enzymes, including prpsT P2, prpsJ, and eda were genomically inserted into the place of wild-type genes from Z. mobilis. E. coli strains B00139, ATCC-8739, W31105, HB101, BL21, JM109, and DH5α were used for the universal verification of phDRC with the same operations. To test the genomic level regulation of phDRC, the N terminus of arroK was tagged with a TEVp site (cleavage site: ENLYFQ) followed by the F-degron (FLVQ). This modified arroK was genomically inserted into the place of wild-type arroK under the control of promoter pRPP P2 in chassis S4. Pathway enzymes, including arroPΦ, arroB, and arroF were selected for constructing enzyme overexpression plasmid pGABE. The backbone plasmid was a constitutive expression plasmid pJ01 (GenBank accession MK234483) with a pMB1 replication origin. The gene arroCΦ, a feedback-resistant mutant involving D146N, was directed mutagenesis. The gene fltK was codon-optimized variant, was obtained by optimization of the first eight codons (ATG GAG GGT ATG GTC ATT CTG CTG). An improved solubility and autolysis inactive TEVp mutant (T17S, xylB C. crescentus CTG) was obtained by rapid PCR site-directed mutagenesis. The gene fltKΦ was optimized after the coding sequence from Takara Bio (Dalian, China) or one-step cloning kit procedure from Vazyme Biotech (Nanjing, China). A list of all generated using ligation cloning procedures from Takara Bio (Dalian, China) or one-step cloning kit procedure from Vazyme Biotech (Nanjing, China). A list of all DNA sequences of those plasmids can be found in Supplementary Data 1. Supplementary Data 1, Supplementary Fig. 20, and Supplementary Tables 2-7. For SHK production, E. coli MG1655 was used for chassis strain S4 construction. The gene aroE was genomically replaced by a tktA expression cassette under the control of promoter rpsT P2 in chassis S4. Pathway enzymes, including arroPΦ, arroB, and arroF were selected for constructing enzyme overexpression plasmid pGABE. The backbone plasmid was a constitutive expression plasmid pJ01 (GenBank accession MK234483) with a pMB1 replication origin. The gene arroCΦ, a feedback-resistant mutant involving D146N, was obtained by rapid PCR site-directed mutagenesis. The gene fltK was codon-optimized variant, was obtained by optimization of the first eight codons (ATG GAG GGT ATG GTC ATT CTG CTG). An improved solubility and autolysis inactive TEVp mutant (T17S, xylB C. crescentus CTG) was obtained by rapid PCR site-directed mutagenesis. The gene fltKΦ was optimized after the coding sequence from Takara Bio (Dalian, China) or one-step cloning kit procedure from Vazyme Biotech (Nanjing, China). A list of all generated using ligation cloning procedures from Takara Bio (Dalian, China) or one-step cloning kit procedure from Vazyme Biotech (Nanjing, China). A list of all DNA sequences of those plasmids can be found in Supplementary Data 1. Supplementary Data 1, Supplementary Fig. 20, and Supplementary Tables 2-7. For SHK production, E. coli MG1655 was used for chassis strain S4 construction. The gene aroE was genomically replaced by a tktA expression cassette under the control of promoter rpsT P2 in chassis S4. Pathway enzymes, including arroPΦ, arroB, and arroF were selected for constructing enzyme overexpression plasmid pGABE. The backbone plasmid was a constitutive expression plasmid pJ01 (GenBank accession MK234483) with a pMB1 replication origin. The gene arroCΦ, a feedback-resistant mutant involving D146N, was obtained by rapid PCR site-directed mutagenesis. The gene fltK was codon-optimized variant, was obtained by optimization of the first eight codons (ATG GAG GGT ATG GTC ATT CTG CTG). An improved solubility and autolysis inactive TEVp mutant (T17S, xylB C. crescentus CTG) was obtained by rapid PCR site-directed mutagenesis. The gene fltKΦ was optimized after the coding sequence from Takara Bio (Dalian, China) or one-step cloning kit procedure from Vazyme Biotech (Nanjing, China). A list of all generated using ligation cloning procedures from Takara Bio (Dalian, China) or one-step cloning kit procedure from Vazyme Biotech (Nanjing, China). A list of all DNA sequences of those plasmids can be found in Supplementary Data 1. Supplementary Data 1, Supplementary Fig. 20, and Supplementary Tables 2-7.}

Culture conditions. For SHK production in shaker flasks, seed cultures were grown overnight in LB medium at 37 °C and then transferred into 50 mL NBS minimal medium (3.5 g L−1 KH2PO4, 5.0 g L−1 K2HPO4, 3.5 g L−1 (NH4)2HPO4, 0.25 g L−1 MgSO4·7H2O, 15.0 mg L−1 CaCl2·2H2O, 0.5 mg L−1 vitamin B1, 1.0 mL L−1 10× transfer medium, 10.0 mg L−1 F6C, 0.01 mL L−1 1-phenylalanine) and 1× C6H2O (200 mg L−1 glucose, 0.1× CuCl2·2H2O, 0.2 g L−1 ZnCl2, 1.0 g L−1 MgSO4·7H2O, 0.2 g L−1 FeCl3·6H2O, 0.5 g L−1 K2HPO4, 3.5 g L−1 KCl, 20 mg L−1 thiamine, 300 mg L−1 Na2S·9H2O, 0.5 g L−1 1-Mercaptopyrrolidine). After growth at 37 °C for 20 h, the culture was diluted 1:100 in 200 mL of LB medium with 200 ng mL−1 IPTG and 200 mL−1 ATC were added. For time-course determinations, this was the t = 0 h time point. Continuous sampling was performed for fluorescence detection on a SpectraMax M3 microplate reader.

For flow cytometry analysis, sample cells were washed twice with phosphate-buffered saline (PBS) and resuspended to an OD600 of 0.2. The assays were performed using a LSR Fortessa (BD Biosciences) using fluorescein isothiocyanate (FITC) (GFPI and PE-TxRed (mCherry) channels). The voltage gains for each detector were set to FITC 407 V and PE-TxRed, 650 V. Compensation was performed using cells that express only GFP or mCherry. For each sample, at least 10,000 counts were recorded using a 0.5 mL−1 1 flow rate. All data were exported in FCS3 format and processed using FlowJo software (FlowJo, LLC).

Single-cell time-lapse fluorescence microscopy. The molten LB medium was poured onto a glass slide after adding appropriate antibiotics (Amp and Cm) and inducers (IPTG and ATC). This agarose pad was solidified at room temperature. When the density of exponentially growing strains reached 0.8, inducers of IPTG and ATC were added. Then, 1 μL of cultures were pipetted onto an agarose pad and a cover slide was put on the top softly to prevent evaporation. Strain was in situ cultured at 30 °C and this time was set as zero-time point. Microscope images were taken using a Nikon ECLIPSE 80i microscope equipped with a ×100 oil-immersion objective. Phase-contrast and fluorescence time-lapse images were recorded every 30 min using a Nikon DS-R1i camera. Bright-fields images (intensity, 40%; exposure, 30 ms) and GFP fluorescence images (intensity, 40%; excitation, 495 nm; emission, 525/50 nm, exposure, 600 ms) were analyzed using ImageJ software.

Kinetics experiment. For testing the effects of inducing TEVp on the degradation of fluorescent proteins, cultures were first grown in the pre-induction condition in shaker flasks (LB + 0.4% glucose, 0.5 mM IPTG), where target mCherry protein was expressed. Upon the OD600 reaching 0.3 (2 h), 1 mL of culture was sampled, diluted 1:100 in 200 mL of LB media (with 200 ng mL−1 IPTG), and then resuspended in 0.05 M barbital buffer (pH 7.0). Crude extracts were obtained using a F-degron. C. crescentus JM109 cells carrying corre-

SHK kinase activity assay. Strains were cultured in LB medium and collected every 6 or 12 h, centrifuged at 12,000 g for 10 min, washed with cold saline solution, and then resuspended in 3 mL of 4 M guanidine hydrochloride. The resulted extracts were lyzed by sonication and centrifuged at 12,000 g for 10 min. Protein concentration was determined by the Bradford method using bovine serum albumin as a standard. SHKkinase activity 1 was measured in a 1 mL reaction mixture containing 4 μM ATP, 1 μM SHK, 10 μM NaF, 5 μM MgCl2, 25 μM barbitur buffer (pH 9.0), and cell extract with 0.1–1.0 mg of protein. One unit of SHK kinase corresponded to 1 μM of SHK consumed per minute.

Strain vitality and viability test. For vitality staining, 1 ng mL−1 propidium iodide (catalog number P3556; Invitrogen) was added to diluted samples and incubating the strain XO in shaker flasks containing liquid LB medium overnight at 37 °C. Then, 5% (vol vol−1) of seed culture was inoculated into 2 L of the TB medium with 100 mg mL−1 ampicillin in a 5 L INFORS fermenter. The culture was first operated in a batch mode and the control settings were as follows: 37 °C, stirring speed 600 rpm, and airflow rate at 0.5 vvm. During the culture process, the pH was controlled at 7.0 via automated addition of 30% NH4OH and 2 M HCl. Antifoam 204 was added to prohibit foam development. When the dissolved oxygen started to increase at 4 h (OD600 = 14–15), 0.5 mM IPTG, 200 mg mL−1 arbutidretacyclene, and 70 g L−1 d-xylose were added for d-xylose biosynthesis. Moreover, 580 g L−1 glucose was fed in 8 h, at 0.15 mL min−1 glucose 1 h−1 glucose supplementation until the end of culture. When the concentration of d-xylose fell below 20 g L−1, 70 g L−1 d-xylose was added. Samples were taken to monitor cell density, residual sugar, and organic acid accumulation.
incubated for 15 min. Images were acquired using a Nikon Eclipse 80i microscope equipped with a Nikon DS-Ri1 camera. The viability of cells was assessed by inoculating LB plates with cells grown in liquid NBS. Cells were diluted to OD600 = 0.5 and 10x of serially diluted cell suspension was spread on each agar plate. Plates were incubated at 37°C for 12 h before counting.

**pHluorin calibration.** Cells were inoculated on ice for 5 min, centrifuged, and resuspended with cold PBS buffer. The collected cells were incubated at 37°C for 30 min with solutions containing 150 mM KCl, 20 mM nigericin, and 50 mM buffering agents (for pHs ≤ 5.5, sodium acetate; for pHs to 6.5, morpholine-ethane-sulfonic acid; for pHs 7 to 8, phosphate). The ratio (Rf/R0) of pHluorin fluorescence emitted (510 nm) under excitation at 410 and 470 nm was used to measure intracellular pH.

**Analysis of chemical concentrations.** The concentrations of SHK, acetate, glucose, α-xylose, and α-xylonate were quantified with an HPLC system (Agilent 1260 Infinity, USA), equipped with an Anionex HPX-87H ion-exchange column (300 x 7.8 mm, Bio-Rad, USA) and a refractive index detector. Analysis was performed with a mobile phase of 5 mM sulfuric acid (65 °C) at a flow rate of 0.6 mL min⁻¹ and detected by monitoring absorbance at 210 nm.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** Data supporting the findings of this work are available within the paper and its Supplementary Information files. A reporting summary for this Article is available as a Supplementary Information file. The datasets generated and analyzed during the current study are available from the corresponding author upon request. The GenBank accession numbers and the associated hyperlinks of the 14 key plasmids developed in this study are provided in Supplementary Table 7. The source data underlying Fig. 1c–f, i, j, 2b–c, 3c–d, 4, 5b–g, 6b–d, and 7b–e, as well as Supplementary Figure 1b, 2, 3c, 4d–e, 6b, 7b, 8, 9a–b, 11, 12b, 13, 15–19 are provided in Source Data file.

Received: 11 March 2019 Accepted: 1 August 2019
Published online: 21 August 2019

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Acknowledgements
We thank Professor Jens Nielsen from Chalmers University of Technology and Dr. Yujia Qing from University of Oxford for discussions and comments. This work is supported by the National Key R&D Program of China (2018YFA0901401), the National Natural Science Foundation of China (21676118, 21706095, and 21808083), and the National First-class Discipline Program of Light Industry Technology and Engineering (LITE2018-08).

Author contributions
C.G., P.X. and L.M.L. conceived this project and designed the experiments. C.G. and J.S.H. conducted and interpreted all fermentation experiments. L.G., X.L.C. and G.P.H. provided technical assistance. L.M.L. provided overall project supervision. C.G. and J.S.H. analyzed the data and wrote the manuscript, with input from P.X., H.E., C.Y., J.C., W.C. and L.M.L. All authors reviewed and approved the manuscript.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-11793-7.

Competing interests: The authors declare no competing interests.

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Peer review information: Nature Communications thanks the anonymous reviewers for their contribution to the peer review of this work.

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