

Engineering dynamical control of cell fate switching using synthetic phospho-regulons

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Many cells can sense and respond to time-varying stimuli, selectively triggering changes in cell fate only in response to inputs of a particular duration or frequency. A common motif in dynamically controlled cells is a dual-timescale regulatory network: although long-term fate decisions are ultimately controlled by a slow-timescale switch (e.g., gene expression), input signals are first processed by a fast-timescale signaling layer, which is hypothesized to filter what dynamic information is efficiently relayed downstream. Directly testing the design principles of how dual-timescale circuits control dynamic sensing, however, has been challenging, because most synthetic biology methods have focused solely on rewiring transcriptional circuits, which operate at a single slow timescale. Here, we report the development of a modular approach for flexibly engineering phosphorylation circuits using designed phospho-regulon motifs. By then linking rapid phospho-feedback with slower downstream transcription-based bistable switches, we can construct synthetic dual-timescale circuits in yeast in which the triggering dynamics and the end-state properties of the ON state can be selectively tuned. These phospho-regulon tools thus open up the possibility to engineer cells with customized dynamical control.

dynamical control | synthetic biology | phosphorylation

Long-term cell fates can often be selectively triggered by specific temporal patterns (dynamics) of stimulation (Fig. 1A). Relatively few cellular systems that “decode” time-varying inputs have been characterized in detail, but recurrent network motifs are beginning to emerge (1, 2). One key feature that is often observed in such systems is the interlinking of circuits that operate on distinct timescales (3–14). In perhaps the best example of a biological “dynamic gate,” the synaptic remodeling of neurons is mediated by two layers of regulation (Fig. 1B): first, an upstream circuit of rapid but transient allosteric and posttranslational changes detects incoming stimuli and filters for high-frequency pulses; second, the signal is transmitted to downstream circuits regulated by slower processes (gene expression, trafficking, and morphological changes), which ultimately can yield stable alterations in receptor localization and synaptic function (4, 6). This common motif suggests that a simple solution for achieving tunable dynamic control systems is to link fast and slow subnetworks, whereby the upstream fast system processes how the intrinsically slow downstream switch receives and responds to external dynamic inputs.

To test this hypothesis, we engineered synthetic cellular circuits based on linked fast (phosphorylation)- and slow (gene expression)-timescale modules (Fig. 1C). We first developed a versatile method for building fast-timescale signaling circuits in yeast using modular phospho-regulons. We then linked engineered phospho-feedback circuits with an intrinsically slow downstream transcription-based bistable switch, and were thereby able to generate a dynamic cell fate switch in yeast whose principal behaviors (input pulse length sensitivity and output response amplitude) could be selectively tuned by systematically altering the fast and slow regulatory layers.

To date, most engineered cellular circuits have been constructed from gene expression components, taking advantage of the modular nature of promoters (15–20). Significantly, however, the dynamic properties of transcriptional circuits are intrinsically constrained to the slow timescale of gene expression. In native regulatory networks, more rapid responses are often

mediated by faster posttranscriptional modifications, such as protein phosphorylation. Our ability to create novel phosphorylation-based circuits, however, is far less developed. Successful examples of rewiring kinase pathways have largely involved redirecting kinases or phosphatases preferentially to one of several alternative preexisting substrates via engineered recruitment interactions (19, 21, 22). To construct the kind of fast-timescale feedback circuits observed in many cellular dynamic gates, we first needed to develop a more flexible platform for phospho-engineering—one that links the activity of a particular kinase to arbitrary targets in a manner that predictably alters target activity. Phospho-regulated proteins often contain bifunctional sequences [linear motif switches (23, 24)] that are efficient phosphorylation substrates for the upstream kinase as well as inducible ligands for a downstream effector domain. Here, we used one such bifunctional sequence to generate a set of synthetic modular phospho-regulation tags (phospho-regulons) controlled by Fus3, the mitogen-activated protein kinase (MAPK) of the yeast mating pathway.

Results

Phosphorylation-Regulated Interaction Modules for Fast-Timescale Synthetic Circuits. To create a robust synthetic MAPK phospho-regulon, we started with an existing substrate protein that already contains the desired core regulatory behavior (MAPK-regulated binding), and then minimized the protein to obtain a short modular

Significance

Many long-term cellular decisions in development, synaptic plasticity, and immunity require cells to recognize input dynamics such as pulse duration or frequency. In dynamically controlled cells, incoming stimuli are often processed and filtered by a rapid-acting signaling layer, and then passed to a downstream slow-acting layer that locks in a longer-term cellular response. Directly testing how such dual-timescale networks control dynamical regulation has been challenging because most tools in synthetic biology allow rewiring of slow gene expression circuits, but not of rapid signaling circuits. In this work, we developed modular peptide tags for engineering synthetic phosphorylation circuits. We used these phospho-regulons to build synthetic dual-timescale networks in which the dynamic responsiveness of a cell fate decision can be selectively tuned.

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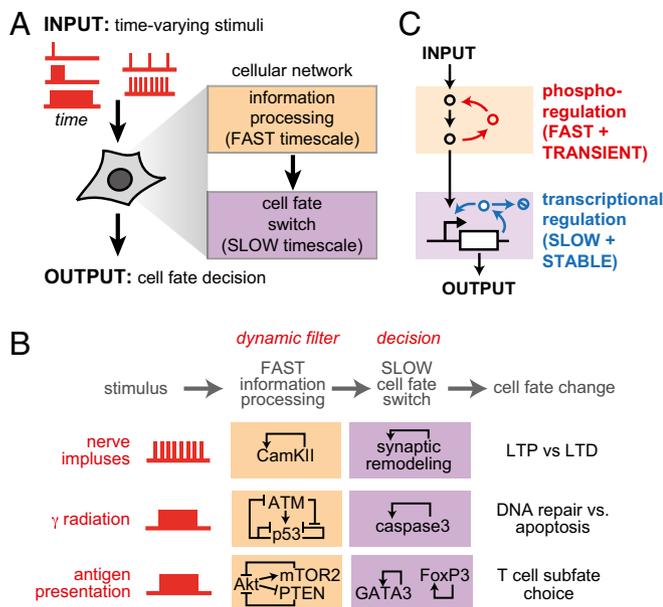


Fig. 1. Dual-timescale architecture is a common feature of regulatory networks that dynamically control cell fate decisions. (A) Dual-timescale regulation (fast and slow layers) has been implicated as an important regulatory mechanism in cellular dynamic gates. (B) This two-layer network architecture is shared by cell fate decision circuits used in neural plasticity (3, 4), apoptosis (7, 8), and adaptive immunity (9, 10). (C) Design principles of a dual-timescale dynamic gate—rapid timescale layer processes and filters inputs, determining what temporal patterns will be propagated to the slow-timescale memory layer.

sequence that can be readily fused to any target protein to achieve phospho-regulation (Fig. 2A and Fig. S1). The yeast mating pathway is one of the best-studied signal transduction networks in eukaryotes, and our work capitalized on the current molecular understanding of fast-timescale regulation by its associated MAP kinase, Fus3. The transcription factor Tec1 is a Fus3 (MAPK) substrate that, when phosphorylated, binds the WD40 domain of Cdc4 (an SCF ubiquitin ligase complex adaptor protein) (25). To generate a minimal WD40 phospho-binding motif, we combined a short, 11-residue peptide from Tec1 that, when phosphorylated, binds to the WD40 domain, with a well-characterized Fus3 docking motif (from the MAPKK Ste7) (26) to form a 49-residue phospho-regulon tag. In an *in vitro* binding assay, we found that this phospho-interaction module indeed only binds the Cdc4 WD40 domain when phosphorylated by the MAPK Fus3 (Fig. 2B). Consistent with this model, mutation of key phosphorylation sites in the synthetic module (Thr→Val) disrupted the Fus3-induced interaction in this and other related phospho-regulon constructs.

To confirm that the synthetic interaction tag mediated rapid, phospho-mediated binding *in vivo*, we fused three tandem copies of the synthetic phospho-regulon to a fluorescent reporter (mCerulean) tagged with a plasma membrane targeting sequence (CAAX motif), and fused two copies of the cognate Cdc4 WD40 domain to a cytoplasmic fluorescent reporter (tdTomato). Under basal conditions, the tdTomato reporter protein is distributed throughout the cytoplasm. Once cells have been stimulated with α -factor (the yeast mating pheromone that activates the Fus3 MAPK), Fus3 phosphorylates the motif, triggering rapid recruitment of the cytoplasmic reporter to the plasma membrane in ~ 5 min (Fig. 2C and Figs. S2A and S3A; optimization described in *SI Materials and Methods*).

The modular phospho-regulon approach can be readily adapted for alternative modes of posttranslational regulation: phospho-regulated degradation and changes in nuclear/cytoplasmic distribution. We assembled a modular phospho-degron (Fig. 2D and Figs. S2B and S3B and C) using an extended region of Tec1 [characterized by Bao et al. (25)] that (i) is phosphorylated by the Fus3 MAPK, (ii) inducibly binds the endogenous yeast Cdc4 E3

ubiquitin ligase complex, and (iii) contains an additional polylysine region that facilitates ubiquitylation. Although the fusion of this initial phospho-degron to a fluorescent reporter (tdTomato) showed slow degradation kinetics, mutational optimization of the Cdc4 binding region readily yielded an improved variant that rapidly degraded the reporter protein upon MAPK activation ($t_{1/2} = 14$ min) (*SI Materials and Methods* and Fig. S2B). Similarly, we constructed a localization control module by fusing the phospho-degron to a nuclear anchor protein, which then released a fluorescent reporter protein into the cytoplasm upon MAPK stimulation (Figs. S2 and S3). These experiments illustrate the flexibility of phospho-regulons for circuit engineering.

To create a synthetic fast-timescale positive-feedback loop, which could alter the dynamics of a response, we used our original phospho-interaction module to drive colocalization of the mating pathway proteins Ste18 (tagged with the Fus3 phospho-regulon) with the Ste50 SAM domain (linked to the Cdc4 WD40 phospho-binding domain)—thereby reconstituting a potent synthetic activator of Fus3 MAPK [tethering of Ste18 and Ste50 SAM has been shown to mediate formation of the active MAPK signaling complex (27)] (Fig. 2E). Positive phospho-feedback reshaped the mating pathway response dynamics such that cells stimulated with pheromone displayed significantly accelerated expression of a downstream transcriptional reporter (pFus1-GFP) relative to cells containing either (i) no phospho-feedback or (ii) a circuit in which phospho-threonine residues in the recruitment module were mutated to valine. This accelerated response was correlated with rapidly amplified Fus3 phosphorylation, assayed by Western blot (Fig. S4). Notably, the synthetic phospho-regulated feedback loop did not lead to bistable memory behavior (i.e., persistent activation after stimulus removal).

To engineer a fast-timescale negative-feedback loop, we used the phospho-recruitment module to colocalize components of a complex previously shown to inhibit mating pathway activation [Fus3 and the N-terminal domain of Ste20 (27)]. In this case, we observed the opposite change in pathway behavior—deceleration of pheromone induced GFP-reporter expression (Fig. S5).

Combination of Phospho-Regulation and Transcriptional Regulation to Form Dual-Timescale Switches.

To construct a synthetic dual-timescale circuit, we then linked our engineered phospho-circuits to a slower timescale downstream switch. One of the most common and well-studied classes of synthetic switch circuits is the bistable transcriptional memory circuit—a regulatory switch that flips from one stable state to another when perturbed by a transient stimulus pulse of sufficient amplitude and duration. Bistability can be achieved using cooperative positive feedback (28), and several synthetic memory circuits have previously been constructed using autoregulated transcription factors (15–18, 29). These gene expression switches are intrinsically slow and can take hours or more of stimulation to trigger the transition from the low-expression to high-expression steady state. To understand how changes in the upstream kinase circuit could alter dynamic gating behavior, we created a simple deterministic computational model of a dual-timescale system composed of sequential phosphorylation and transcriptional feedback loops (Fig. 3A; simulation details described in *SI Materials and Methods*). Based on this model, a circuit with an added fast positive-feedback loop was predicted to switch ON in response to shorter stimulus pulses than a circuit with no upstream feedback. In time course simulations, the addition of positive phospho-feedback enhanced the amplitude of stimulus-dependent kinase activation, which, in turn, accelerated the rate of transcription factor synthesis, thereby decreasing the stimulus duration required to cross the bistable circuit's threshold for self-sustaining activation. Conversely, addition of a negative phospho-feedback loop was predicted to delay the circuit's commitment to switching ON (increase triggering time).

Experimentally Tuning the Trigger Time of a Dual-Timescale Bistable Switch.

We then experimentally examined the role of fast-timescale feedback in dual-timescale switches, constructing sequential fast/slow-feedback circuits (Fig. 3A) by linking synthetic phospho-feedback on

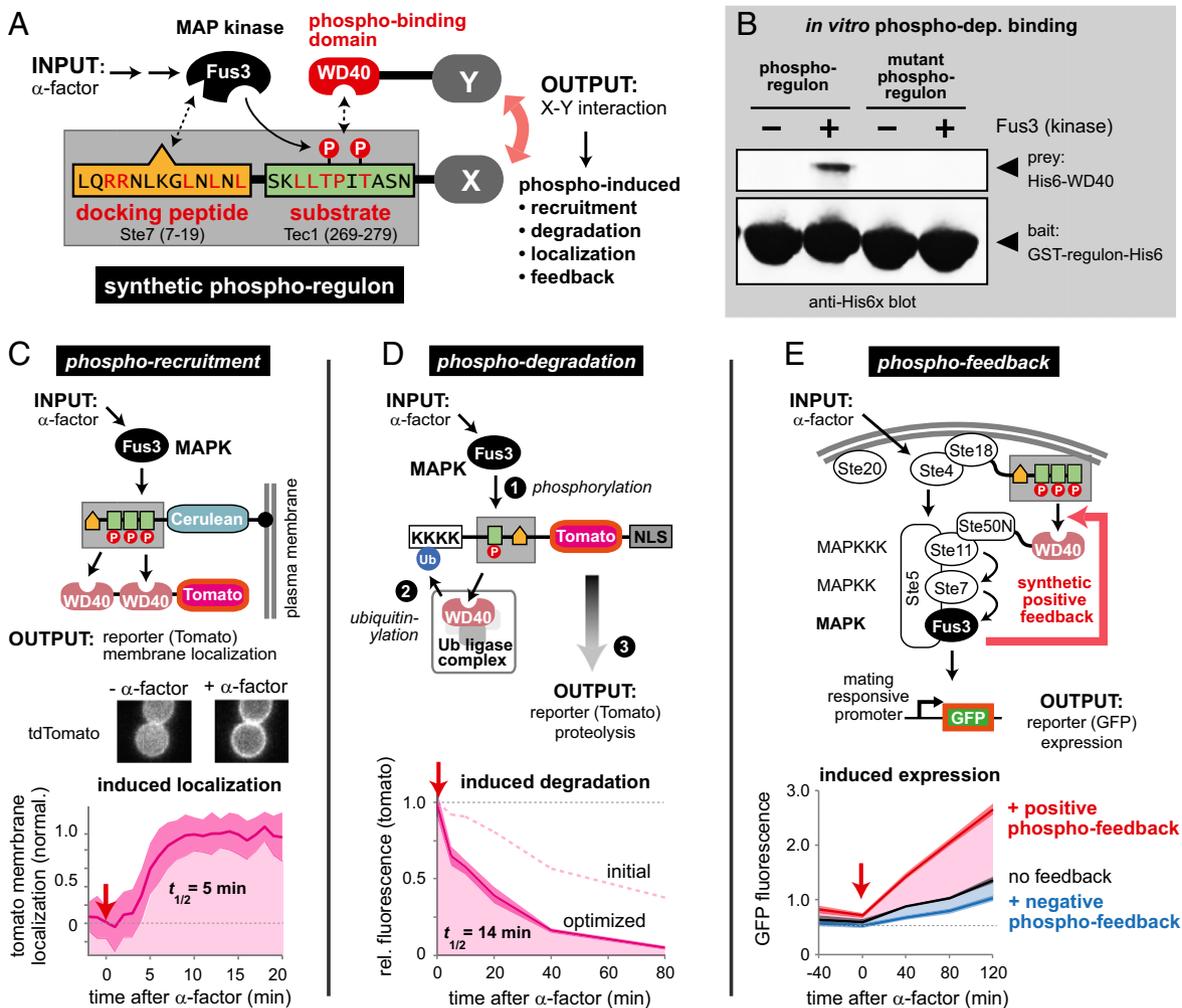


Fig. 2. Design of phospho-regulation modules that allow engineering of synthetic kinase–substrate relationships. (A) A synthetic phospho-regulated interaction module in which phosphorylation by a yeast MAP kinase (Fus3) triggers recruitment of a phospho-binding domain (WD40 domain of Cdc4). The synthetic phospho-regulon is composed of composite linear motifs optimized for three simultaneous recognition functions: MAPK docking, MAPK phosphorylation, and phospho-binding domain recognition. Key recognition residues are denoted in red. (B) GST pull-downs confirmed phospho-dependent binding of the synthetic phospho-regulon components *in vitro*. (C) Phospho-regulated plasma membrane recruitment, in yeast, triggered by mating pathway activation (α -factor). “+ α -factor” image taken 20 min after induction. Time course of tdTomato reporter membrane recruitment, quantified by image analysis; mean \pm SD ($n = 54$ cells; shaded region) are shown. (D) The phospho-regulon can be converted to a phospho-dependent degradation motif (induced with α -factor) by adding lysine (K) residues to serve as ubiquitinylation sites. Phosphorylation by Fus3-triggered binding by endogenous E3 ligase Cdc4 [a substrate targeting component of the SKP1–CUL1–F-box protein (SCF) ubiquitin ligase complex (25)], ubiquitination, and proteolysis. Fus3-induced degradation was enhanced by screening mutant phospho-regulons and combining enhancing mutations (Fig. S2). Time course of reporter decay in yeast after α -factor stimulation is shown (RFP fluorescence measured by flow cytometry, normalized to cell volume; see *Materials and Methods*). Mean \pm SD ($n = 3$; shaded region) are shown. (E) Phospho-regulons can be used to build synthetic fast positive and negative feedback into the yeast mating pathway. Positive feedback was generated by using Fus3 phospho-regulon to induce interaction of Ste18 and Ste50N; this complex acts as a positive regulator of pathway activity, increasing the extent of Fus3 phosphorylation and downstream transcription (pFus1-GFP reporter). An analogous negative phospho-feedback loop was engineered by using the phospho-regulon to induce formation of a known inhibitory complex [Fus3 and Ste20, Fig. S5 (27)]. GFP fluorescence (measured by flow cytometry) is normalized to cell volume (*Materials and Methods*). Mean \pm SD ($n = 3$; shaded region) are shown. Phospho-regulons mutated to prevent phosphorylation (pT \rightarrow V) do not mediate *in vivo* recruitment, degradation, or feedback (Figs. S3–S5).

the MAPK Fus3 (from Fig. 2E and Fig. S5) to a transcription-based memory circuit. The transcriptional memory switch has the following components: (i) a promoter activated by Fus3 (pFig2) drives production of an artificial transcription factor (VP64-LexA DNA binding domain), (ii) a promoter activated by the transcription factor (LexA Operator-pGal) drives production of a fluorescently tagged version of the transcription factor (tdTomato-VP64-LexA DNA binding domain) creating a self-perpetuating positive-feedback loop (Fig. S6; Movie S1 shows the memory persistence of this switch across multiple cell divisions). To measure the trigger time required for commitment to memory formation, we induced the mating pathway (by adding

the peptide α -factor) for varied periods of time before quenching stimulation (by adding Pronase, a mixture of proteases that degrades α -factor) (Fig. 3B). After cells were cultured for an additional 3 h [to allow time for memory consolidation (Fig. S6)—a process that remains slow even if commitment occurs much faster], we then measured the concentration of fluorescently tagged transcription factor in single cells by FACS ($\geq 10,000$). Cells that did not pass the threshold for memory activation returned to basal tdTomato levels after 3 h (due to dilution by cell division) (Fig. 3C). In agreement with our simulations, yeast cells containing the additional negative phospho-feedback module required a longer pulse to trigger memory formation (trigger times of 88 vs. 54 min). Cells containing

weak, DegronE; or strong, DegronR (30)] were stimulated with a prolonged (3-h) pulse of α -factor, a 54-fold range of ON states was observed. (Fig. 4A and Fig. S8).

Thus, our theoretical and experimental analysis suggested that dual-timescale switches were highly modular, such that rewiring of fast and slow regulatory layers could selectively and independently tune switch dynamic sensitivity (to pulse length) and response (amplitude of activated state). In principle, this feature would enhance the plasticity of native cellular dynamic gates and facilitate the design of diverse synthetic memory circuits. To experimentally test this possibility, we constructed a small combinatorial library of dual-timescale switches, combining fast-timescale phospho-feedback loops with downstream transcriptional feedback mediated by transcription factors with high, medium, or low stability (destabilization performed with *N*-end rule degrons). These synthetic circuits exhibited a broad spectrum of trigger times (7–125 min) and ON state amplitudes (84-fold range). As predicted, changes in phospho-feedback selectively modulated duration sensitivity, whereas variations transcription factor stability predominantly tuned the memory response (Fig. 4B and Fig. S8). Notably, however, we did observe a moderate increase in dynamic sensitivity when circuits contained the most stable transcription factor. In this case, it is possible that sustained positive feedback may effectively extend the duration of each input pulse.

Because changes in the strength or sign of phospho-feedback have little impact on the steady state (response amplitude) of the transcription factor-mediated memory, these fast-feedback circuits function analogously to enzymes in that they change the dynamics of switch flipping but do not alter the end states of the switch (Fig. 4C). Thus, the functional modularity of the dual-timescale regulatory switch enables cells to tailor their decision making to the environment (through changes in fast-timescale regulation) without altering, compromising, or destabilizing their long-term response (encoded by the slower downstream switch).

Discussion

Although it is increasingly clear that the interplay between regulatory mechanisms that operate on different timescales plays a central role in the dynamical control of cell fate decisions, the vast majority of synthetic circuits published to date are based on transcriptional regulation. Thus, our ability to understand and engineer dynamical response control is relatively primitive. The development of synthetic phospho-regulons—modular peptide tags built from linear motifs, which create customized phospho-dependent, protein–protein interactions (Fig. 2)—now makes it possible to reroute kinase signaling to directly modulate protein interaction, localization, and stability. These phospho-regulons, as demonstrated here, provide us with synthetic tools for flexibly rewiring fast-timescale cell control circuits. Using phospho-regulons, we show that a simple combination of fast and slow regulation (sequential phosphorylation-based and transcription-based feedback loops) can give rise to tunable cellular switches that are activated by custom-tuned time-varying inputs (dynamic gates). Because time-scale separation permits selective control of switch sensitivity and memory response amplitude (Fig. 4), we anticipate that dual-timescale dynamic gates will be used to build switches that record the dynamics (e.g., duration or frequency) of cellular events. Dynamic gate circuits could also be used to precisely control engineered cells using information-rich modalities [such as pulse sequences (31)] or “sender” cells that encode information in time-varying outputs (32). These tools should help us to better understand the fundamental ways in which cells can encode and decode temporal information.

Materials and Methods

Strain and Plasmid Construction. Details for all assembly protocols, plasmids, and strains (including the transcriptional memory module) are described in *SI Materials and Methods* and Table S1.

GST Pull-Down Binding Experiments. Standard methods were used for expression, purification, and *in vitro* phosphorylation of the phospho-regulon (details in *SI Materials and Methods*). Pull-down experiments (Fig. 2B) were

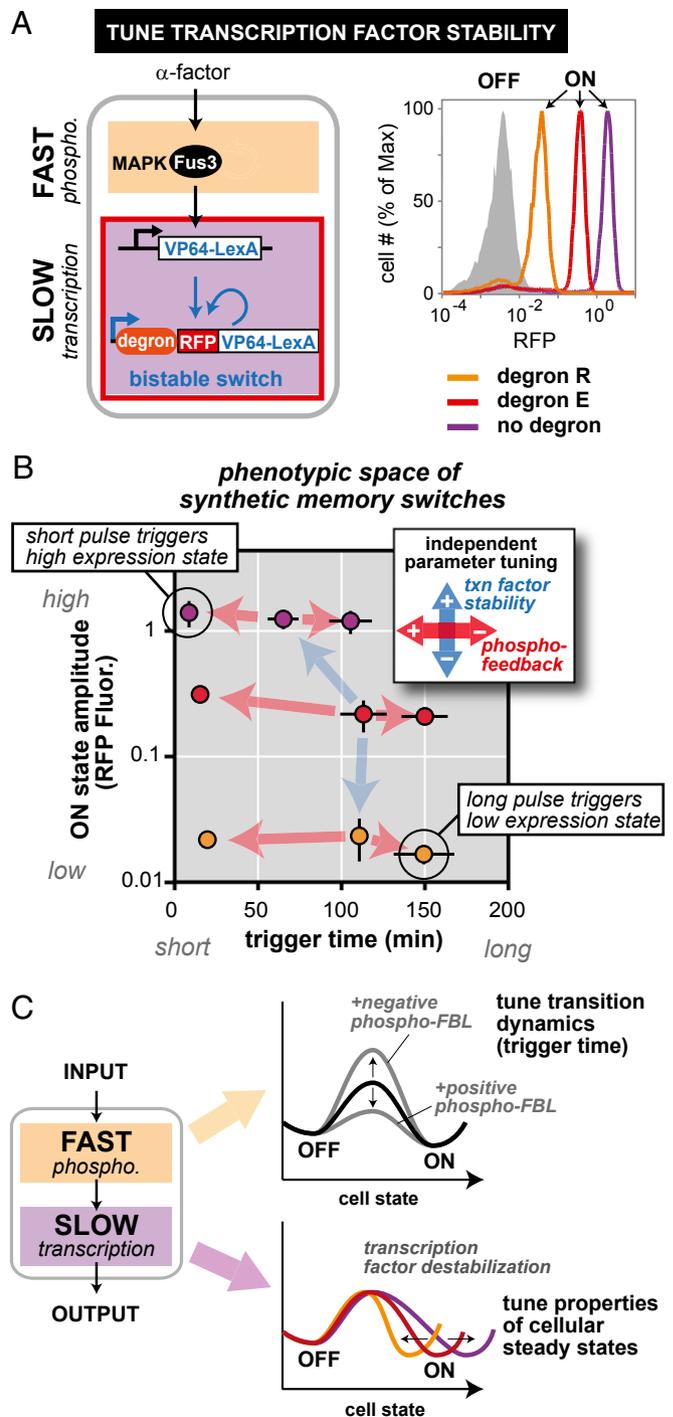


Fig. 4. Independent tuning of cell fate switch dynamics and end states. (A) The ON-state steady-state amplitude of the dual-timescale circuit can be tuned by solely changing the rate of the autoregulatory transcription factor degradation [here, by adding *N*-end rule degron motifs: DegronE (weak) and DegronR (strong) (30)]. Normalized histograms contain >10,000 cells. (B) A small matrix of dual-time circuits illustrates how the trigger time and the steady-state amplitude of the memory ON state can be independently tuned. Changing ON state by altering stability of the transcription factor does not significantly change trigger time. Tuning trigger time by adding phospho-feedback does not significantly change ON-state amplitude. Trigger times and ON-state amplitude calculated as described in *SI Materials and Methods*; mean \pm SD ($n = 3$) are shown. (C) Tuning the fast layer of a dual-timescale switch functions analogously to a catalyst by accelerating the stimulus time required to trigger cell fate change, but without changing the end OFF and ON states. Conversely, destabilizing the autoregulated transcription factor in the slow layer shifts the ON steady state but has little effect on the triggering dynamics.

conducted by incubating GSH-agarose resin harboring ~250 μg of phosphorylated bifunctional motifs with 10–50 μg of the appropriate reader domain. After extensive washing with binding buffer, proteins were eluted from the resin by boiling in SDS/PAGE buffer. Samples were run on an SDS/PAGE gel, and Western blotting was used to detect binding of reader domains. For detection of WD40 binding, mouse anti-His-tag (Cell Signaling; 2366) was used. With primary antibodies, an IRDye 800CW-conjugated IgG (Li-Cor) was used, and blots were imaged on an Odyssey infrared imager (Li-Cor).

Microscopy and Data Analysis. To assay the kinetics of phospho-recruitment of tdTomato to the plasma membrane (Fig. 2D), strains were grown overnight in synthetic complete medium and diluted 1:100 in the morning, and then grown 3–4 h before beginning microscopy in Con A-coated 384-well plates. RFP and CFP epifluorescent images were collected once per minute for 23 min, with α -factor (1 μM final concentration) added after the third minute. Cells were identified by thresholding both fluorescent channels, and membrane translocation of cytoplasmic WD40-tdTomato was computed at each time point as the correlation between the measured intracellular distributions of (phospho-regulon)-mCerulean-CAAX and WD40-tdTomato. Additional details for the microscopy setup and analysis described in *SI Materials and Methods*.

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Flow Cytometry. Full protocols for the flow cytometry assays and analysis are described in *SI Materials and Methods*. Briefly, for α -factor experiments, triplicate 500- μL yeast cultures in log phase were induced for variable periods of time [1 μM α -factor (Zymo Research), removed by adding 0.2 $\mu\text{g}/\text{mL}$ Pronase (Roche)], with aliquots arrested with cycloheximide (allowing time for fluorophore maturation) before analysis with a BD LSR-II flow cytometer. Analysis of flow cytometry data were performed using FlowJo (Tree Star). The fluorescence of each cell was normalized to cell volume (n.f.) in the manner described by Stewart-Ornstein et al. (33).

Quantitative Modeling. Simulations were performed with the free software package GNU Octave. Computational model equations are described in *SI Materials and Methods*.

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