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

Russell M. Gordley[a,b], Reid E. Williams[b,c], Caleb J. Bashor[b,c,1], Jared E. Toettcher[a,2], Shude Yan[b,3], and Wendell A. Lim[a,b,4]

[a]Howard Hughes Medical Institute, San Francisco, CA 94158; [b]Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA 94158; and [c]Graduate Group in Biophysics, University of California, San Francisco, CA 94158

Edited by Eric N. Olson, University of Texas Southwestern Medical Center, Dallas, TX, and approved October 11, 2016 (received for review July 8, 2016)

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. 1*A*). 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. 1*B*): 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. 1*C*). 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.

Author contributions: R.M.G. and W.A.L. designed research; R.M.G., R.E.W., C.J.B., J.E.T., and S.Y. performed research; R.M.G., R.E.W., and J.E.T. analyzed data; and R.M.G., R.E.W., and W.A.L. wrote the paper.

Conflict of interest statement: W.A.L. is a founder of Cell Design Labs and a member of its scientific advisory board.

This article is a PNAS Direct Submission.

[1]Present address: Institute for Medical and Engineering Science, Massachusetts Institute of Technology, Cambridge, MA 02139.

[2]Present address: Department of Molecular Biology, Princeton University, Princeton, NJ 08544.

[3]Present address: Jiangsu Hengrui Medicine, Pudong New District, Shanghai 200122, China.

[4]To whom correspondence should be addressed. Email: wendell.lim@ucsf.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1610973113/-/DCSupplemental.

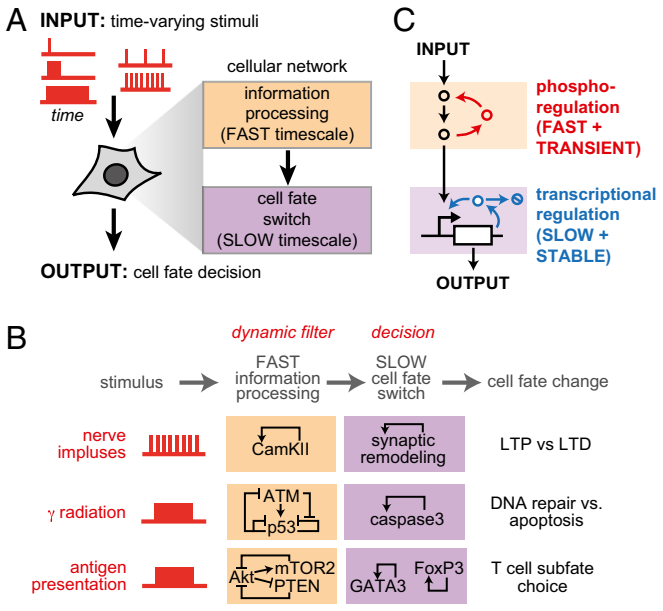

Fig. 1. Dual-timescale architecture is a common feature of regulatory networks that dynamically control cell fate decisions. (*A*) Dual-timescale regulation (fast and slow layers) has been implicated as an important regulatory mechanism in cellular dynamic gates. (*B*) This two-layer network architecture 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. 2*A* 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. 2*B*). 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. 2*C* and Figs. S2*A* and S3*A*; 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. 2*D* and Figs. S2*B* and S3 *B* 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 ubiquitinylation. 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. S2*B*). 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. 2*E*). 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. 3*A*; 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. 3*A*) by linking synthetic phospho-feedback on

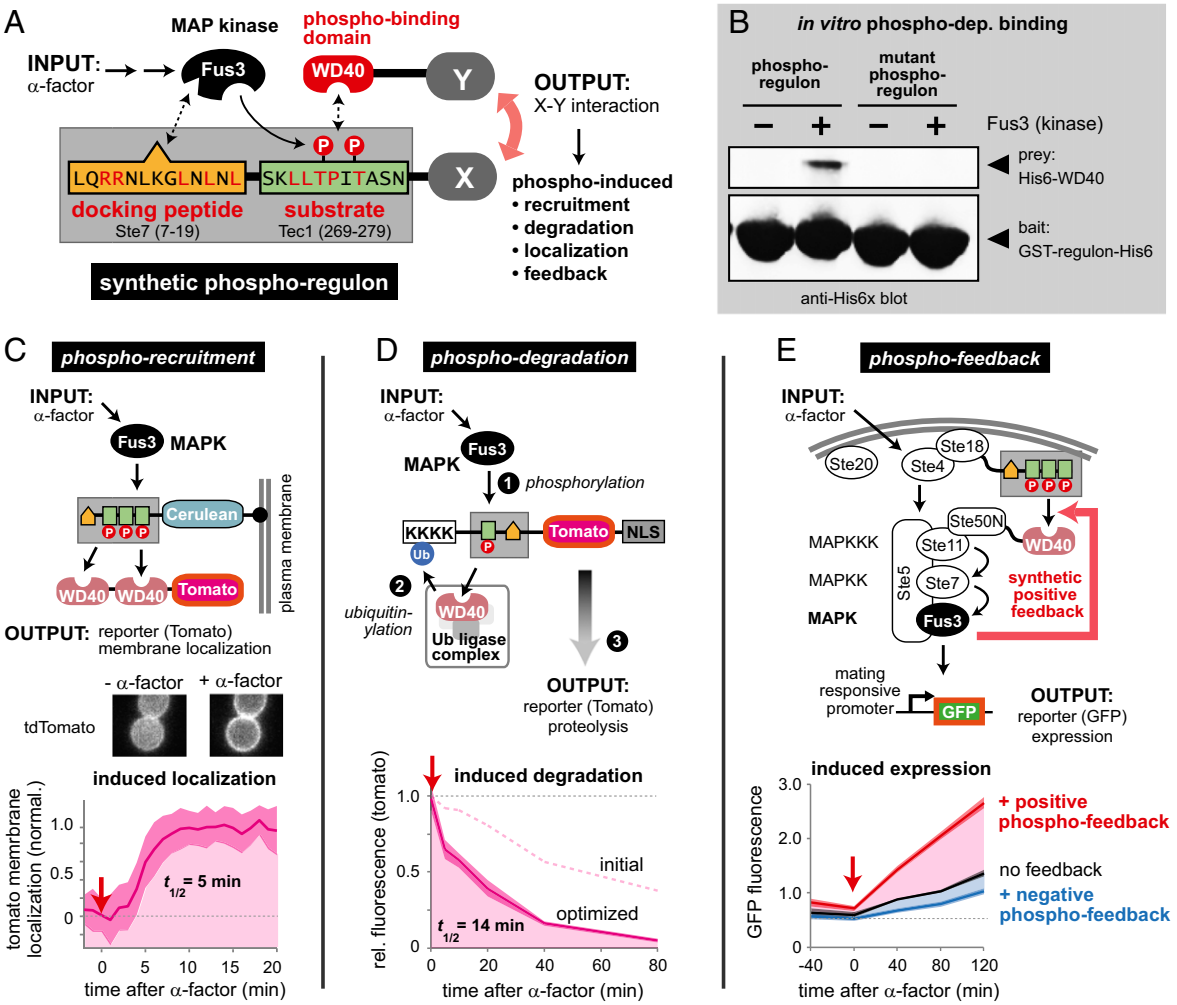

Fig. 2. Design of phospho-regulation modules that allow engineering of synthetic kinase–substrate relationships. (*A*) A synthetic phospho-regulated interaction module in which phosphorylation by a yeast MAP kinase (Fus3) triggers recruitment of a phospho-binding domain (WD40 domain of Cdc4). The synthetic 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 ± 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 ± 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 ± SD (n = 3; shaded region) are shown. Phospho-regulons mutated to prevent phosphorylation (pT→V) do not mediate in vivo recruitment, degradation, or feedback (Figs. S3–S5).

the MAPK Fus3 (from Fig. 2*E* 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. 3*B*). 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 (≥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. 3*C*). 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

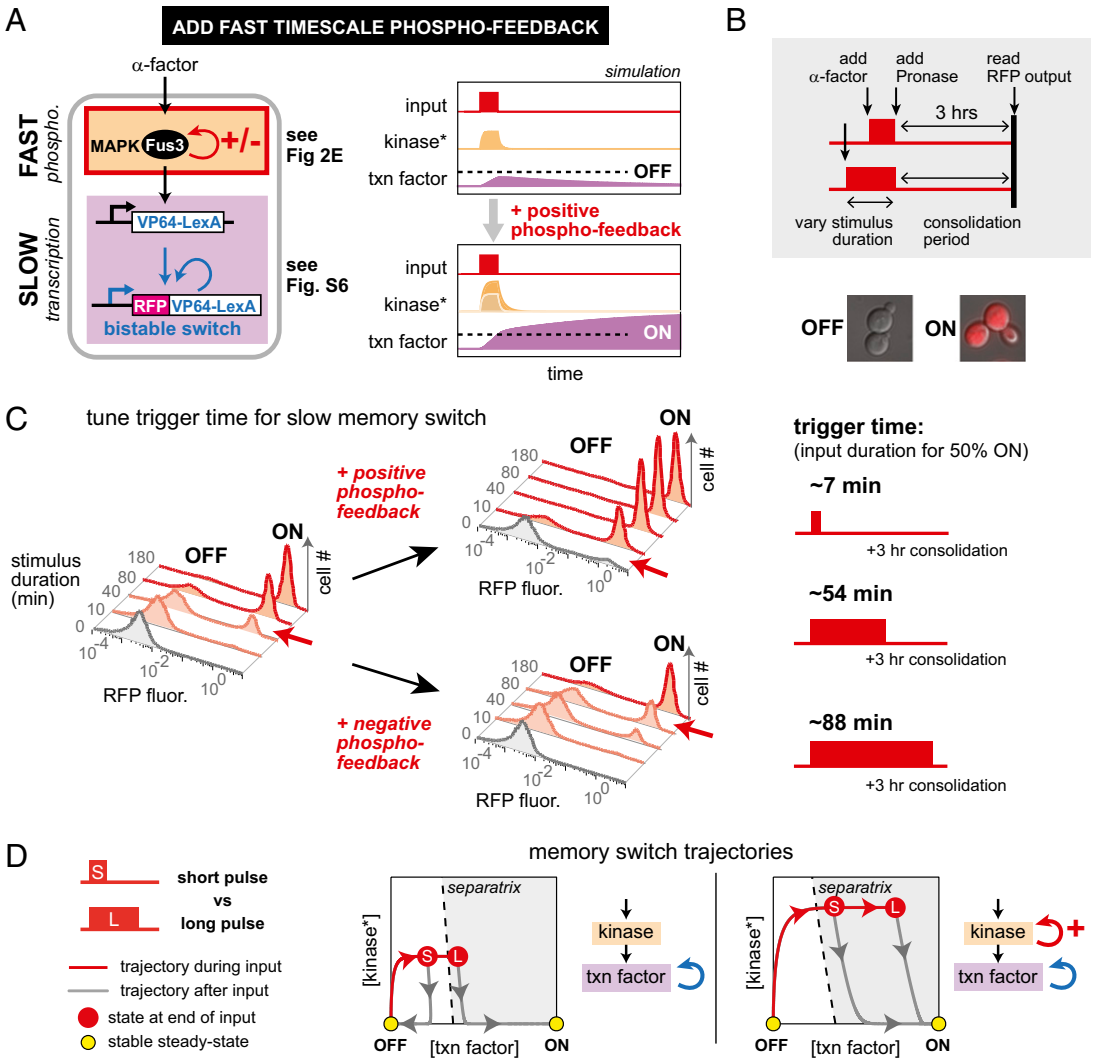

Fig. 3. Engineering fast-timescale phospho-feedback yields yeast cell memory switch with highly tunable switching dynamics. (*A*) Design of a dual-timescale memory circuit: a core transcriptional bistable switch (with autoregulatory transcriptional positive feedback mediated by an synthetic fusion protein combining the VP64 activation domain and LexA DNA-binding domain) is combined with an upstream rapid-timescale MAPK signaling network that incorporates synthetic phospho-feedback loops. In deterministic computer simulations of the dual-time switch, the system flips from OFF to ON when the input duration is sufficient to build up enough transcription factor to pass the threshold for memory formation (dotted line). Addition of fast-timescale positive feedback accelerates the speed with which the system crosses this commitment threshold. (*B*) Pulses of varied duration can be experimentally applied to the mating pathway by adding α-factor and subsequently removing it by rapid proteolytic digestion (Pronase). After a 3-h memory consolidation phase (after end of input pulse), two cell populations emerge: memory ON cells with high levels of tdTomato (RFP), and OFF cells with basal fluorescence. ON cells stay RFP$^+$ for >10 generations (Fig. S6). (*C*) Measuring trigger times for memory switch upon addition of synthetic positive or negative phospho-feedback. Normalized histograms contain >10,000 cells. Trigger times calculated as described in *SI Materials and Methods*, indicated by red arrows. Memory response are shown after 3-h consolidation time after transient exposure to α-factor. Addition of phospho-feedback tunes pulse trigger times by over an order of magnitude. (*D*) Phase plane diagrams depicting trajectories of kinase activation and transcription factor expression in response to short- and long-stimulus pulses (including how the cells relax after the end of the stimulus plus). Addition of fast phospho-feedback dramatically accelerates buildup of active kinase and leads to more rapid crossing of the commitment threshold (separatrix).

positive phospho-feedback module, however, were sensitized to extremely short pulses of α-factor (trigger times of 7 vs. 54 min). Thus, changes in upstream fast-timescale regulation can tune the memory switch trigger time over a 10-fold range, whereas the end states of the switch are unaltered (Fig. 3*C*).

The functional consequences of rewiring either fast or slow layer are evident in phase-plane diagrams of transcription factor concentration and activated kinase concentration (Fig. 3*D* and Fig. S7). Comparison of simulated trajectories for dual-timescale circuits with and without fast positive feedback shows that addition of upstream processing impacts the time evolution of the system during the stimulus pulse—accelerating transcription factor synthesis through enhanced kinase activation—without altering the ON and OFF steady states that the system relaxes to.

Independent Tuning of Switch Dynamics and Steady-State Endpoints. Having observed that feedback in the fast layer selectively tunes the stimulus duration required to trigger the dual-timescale switch, we wondered whether modifications in the slow layer could act as an orthogonal dial, modulating a separate property of the memory switch behavior. When we extended our computational analysis to perturbations in the slow-feedback loop components, we found that variation in the rate of transcription factor loss (degradation plus dilution) alters the position of the switch's end state (i.e., the amplitude of the circuit's memory response) while having relatively little effect on the trigger time (Fig. S7). We experimentally tested this prediction by destabilizing the autoregulated transcription factor in our synthetic memory circuit using *N*-end rule degrons. When cells containing different strength degrons [none;

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. 4*A* 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. 4*B* 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. 4*C*). 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. 2*B*) were

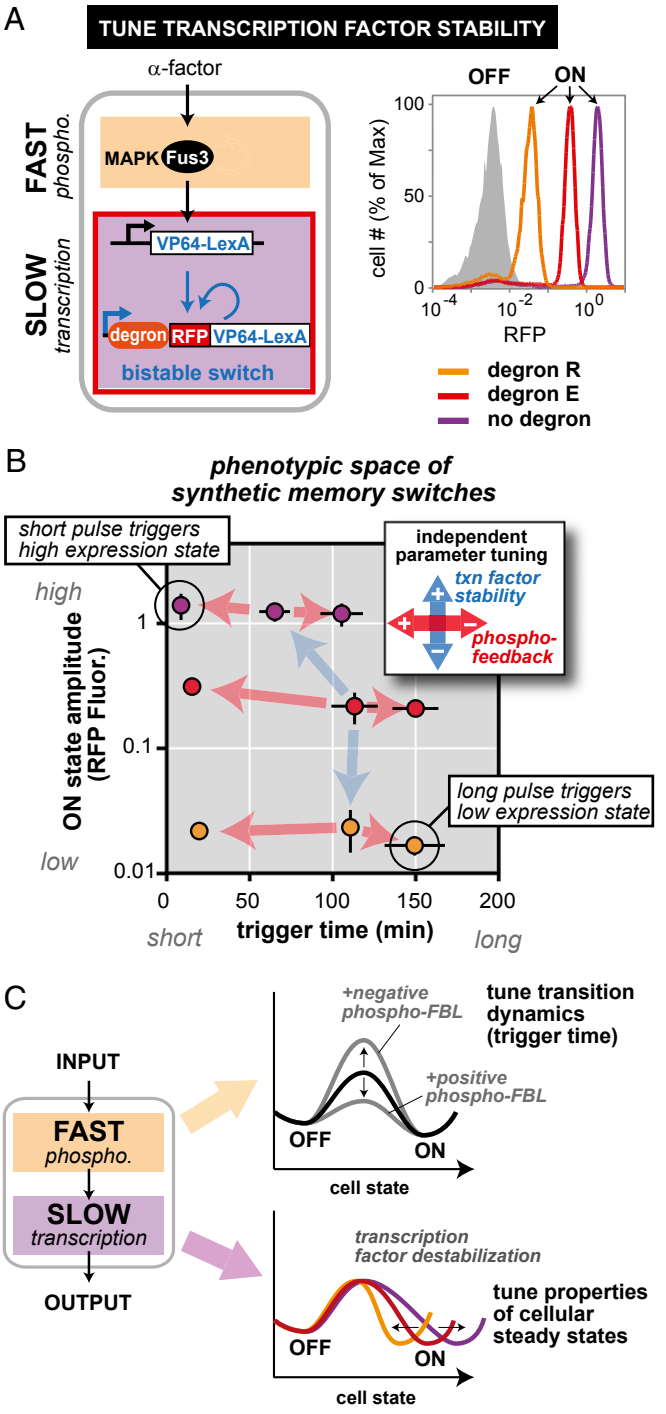

Fig. 4. Independent tuning of cell fate switch dynamics and end states. (*A*) The ON-state steady-state amplitude of the dual-timescale circuit can be tuned by solely changing the rate of the autoregulatory transcription factor degradation [here, by adding *N*-end rule degron motifs: DegronE (weak) and 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 ± 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 coincubating 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. 2*D*), 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*.

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 μg/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*.

ACKNOWLEDGMENTS. We are grateful for the support of the Octave development community. This work was supported by a Ruth L. Kirschstein National Research Service Award (to R.M.G.), a University of California, San Francisco/Genentech Graduate Fellowship (to R.E.W.), a Cancer Research Institute Postdoctoral Fellowship (to J.E.T.), a Jane Coffin Childs Fund Postdoctoral Fellowship (to S.Y.), the National Science Foundation Synthetic Biology and Engineering Research Center (R.M.G. and W.A.L.), the Howard Hughes Medical Institute (R.M.G. and W.A.L.), and National Institutes of Health Grants GM55040, GM62583, EY016546, and GM081879.

1. Purvis JE, Lahav G (2013) Encoding and decoding cellular information through signaling dynamics. *Cell* 152(5):945–956.
2. Yosef N, Regev A (2011) Impulse control: Temporal dynamics in gene transcription. *Cell* 144(6):886–896.
3. Lucchesi W, Mizuno K, Giese KP (2011) Novel insights into CaMKII function and regulation during memory formation. *Brain Res Bull* 85(1-2):2–8.
4. Kandel ER, Dudai Y, Mayford MR (2014) The molecular and systems biology of memory. *Cell* 157(1):163–186.
5. Murphy LO, Smith S, Chen R-H, Fingar DC, Blenis J (2002) Molecular interpretation of ERK signal duration by immediate early gene products. *Nat Cell Biol* 4(8):556–564.
6. West AE, Greenberg ME (2011) Neuronal activity-regulated gene transcription in synapse development and cognitive function. *Cold Spring Harb Perspect Biol* 3(6): a005744.
7. Batchelor E, Loewer A, Mock C, Lahav G (2011) Stimulus-dependent dynamics of p53 in single cells. *Mol Syst Biol* 7:488.
8. Zhang X-P, Liu F, Cheng Z, Wang W (2009) Cell fate decision mediated by p53 pulses. *Proc Natl Acad Sci USA* 106(30):12245–12250.
9. Hawse WF, et al. (2015) Cutting Edge: Differential regulation of PTEN by TCR, Akt, and FoxO1 controls $CD4^+$ T cell fate decisions. *J Immunol* 194(10):4615–4619.
10. Zhu J, Yamane H, Paul WE (2010) Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol* 28:445–489.
11. Miskov-Zivanov N, Turner MS, Kane LP, Morel PA, Faeder JR (2013) The duration of T cell stimulation is a critical determinant of cell fate and plasticity. *Sci Signal* 6(300): ra97.
12. Ribes V, Briscoe J (2009) Establishing and interpreting graded Sonic Hedgehog signaling during vertebrate neural tube patterning: The role of negative feedback. *Cold Spring Harb Perspect Biol* 1(2):a002014.
13. Balaskas N, et al. (2012) Gene regulatory logic for reading the Sonic Hedgehog signaling gradient in the vertebrate neural tube. *Cell* 148(1-2):273–284.
14. Cohen M, et al. (2015) Ptch1 and Gli regulate Shh signalling dynamics via multiple mechanisms. *Nat Commun* 6:6709.
15. Ajo-Franklin CM, et al. (2007) Rational design of memory in eukaryotic cells. *Genes Dev* 21(18):2271–2276.
16. Gardner TS, Cantor CR, Collins JJ (2000) Construction of a genetic toggle switch in *Escherichia coli*. *Nature* 403(6767):339–342.
17. Ingolia NT, Murray AW (2007) Positive-feedback loops as a flexible biological module. *Curr Biol* 17(8):668–677.
18. Kobayashi H, et al. (2004) Programmable cells: Interfacing natural and engineered gene networks. *Proc Natl Acad Sci USA* 101(22):8414–8419.
19. Bashor CJ, Helman NC, Yan S, Lim WA (2008) Using engineered scaffold interactions to reshape MAP kinase pathway signaling dynamics. *Science* 319(5869):1539–1543.
20. Way JC, Collins JJ, Keasling JD, Silver PA (2014) Integrating biological redesign: Where synthetic biology came from and where it needs to go. *Cell* 157(1):151–161.
21. Park S-H, Zarrinpar A, Lim WA (2003) Rewiring MAP kinase pathways using alternative scaffold assembly mechanisms. *Science* 299(5609):1061–1064.
22. Won AP, Garbarino JE, Lim WA (2011) Recruitment interactions can override catalytic interactions in determining the functional identity of a protein kinase. *Proc Natl Acad Sci USA* 108(24):9809–9814.
23. Tompa P, Davey NE, Gibson TJ, Babu MM (2014) A million peptide motifs for the molecular biologist. *Mol Cell* 55(2):161–169.
24. Miller ML, et al. (2008) Linear motif atlas for phosphorylation-dependent signaling. *Sci Signal* 1(35):ra2.
25. Bao MZ, Shock TR, Madhani HD (2010) Multisite phosphorylation of the *Saccharomyces cerevisiae* filamentous growth regulator Tec1 is required for its recognition by the E3 ubiquitin ligase adaptor Cdc4 and its subsequent destruction in vivo. *Eukaryot Cell* 9(1):31–36.
26. Reményi A, Good MC, Bhattacharyya RP, Lim WA (2005) The role of docking interactions in mediating signaling input, output, and discrimination in the yeast MAPK network. *Mol Cell* 20(6):951–962.
27. Peisajovich SG, Garbarino JE, Wei P, Lim WA (2010) Rapid diversification of cell signaling phenotypes by modular domain recombination. *Science* 328(5976):368–372.
28. Ferrell JE, Xiong W (2001) Bistability in cell signaling: How to make continuous processes discontinuous, and reversible processes irreversible. *Chaos* 11(1):227–236.
29. Inniss MC, Silver PA (2013) Building synthetic memory. *Curr Biol* 23(17):R812–R816.
30. Hackett EA, Esch RK, Maleri S, Errede B (2006) A family of destabilized cyan fluorescent proteins as transcriptional reporters in *S. cerevisiae*. *Yeast* 23(5):333–349.
31. Milias-Argeitis A, et al. (2011) In silico feedback for in vivo regulation of a gene expression circuit. *Nat Biotechnol* 29(12):1114–1116.
32. Prindle A, et al. (2014) Rapid and tunable post-translational coupling of genetic circuits. *Nature* 508(7496):387–391.
33. Stewart-Ornstein J, Weissman JS, El-Samad H (2012) Cellular noise regulons underlie fluctuations in *Saccharomyces cerevisiae*. *Mol Cell* 45(4):483–493.
34. Zalatan JG, Coyle SM, Rajan S, Sidhu SS, Lim WA (2012) Conformational control of the Ste5 scaffold protein insulates against MAP kinase misactivation. *Science* 337(6099): 1218–1222.
35. Shao Z, Zhao H, Zhao H (2009) DNA assembler, an in vivo genetic method for rapid construction of biochemical pathways. *Nucleic Acids Res* 37(2):e16.
36. Nevoigt E, et al. (2006) Engineering of promoter replacement cassettes for fine-tuning of gene expression in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 72(8): 5266–5273.
37. Reményi A, Good MC, Lim WA (2006) Docking interactions in protein kinase and phosphatase networks. *Curr Opin Struct Biol* 16(6):676–685.
38. Orlicky S, Tang X, Willems A, Tyers M, Sicheri F (2003) Structural basis for phosphodependent substrate selection and orientation by the SCFCdc4 ubiquitin ligase. *Cell* 112(2):243–256.
39. Voet D, Voet JG, Pratt CW (1999) *Fundamentals of Biochemistry* (Wiley, New York).
40. Chou S, Huang L, Liu H (2004) Fus3-regulated Tec1 degradation through SCFCdc4 determines MAPK signaling specificity during mating in yeast. *Cell* 119(7):981–990.
41. Bao MZ, Schwartz MA, Cantin GT, Yates JR, 3rd, Madhani HD (2004) Pheromone-dependent destruction of the Tec1 transcription factor is required for MAP kinase signaling specificity in yeast. *Cell* 119(7):991–1000.
42. Brückner S, et al. (2004) Differential regulation of Tec1 by Fus3 and Kss1 confers signaling specificity in yeast development. *Curr Genet* 46(6):331–342.
43. Garrison TR, et al. (1999) Feedback phosphorylation of an RGS protein by MAP kinase in yeast. *J Biol Chem* 274(51):36387–36391.