A load driver device for engineering modularity in biological networks

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The behavior of gene modules in complex synthetic circuits is often unpredictable1–4. After joining modules to create a circuit, downstream elements (such as binding sites for a regulatory protein) apply a load to upstream modules that can negatively affect circuit function1,5. Here we devised a genetic device named a load driver that mitigates the impact of load on circuit function, and we demonstrate its behavior in Saccharomyces cerevisiae. The load driver implements the design principle of timescale separation: inclusion of the load driver’s fast phosphotransfer processes restores the capability of a slower transcriptional circuit to respond to time-varying input signals even in the presence of substantial load. Without the load driver, we observed circuit behavior that suffered from a 76% delay in response time and a 25% decrease in system bandwidth due to load. With the addition of a load driver, circuit performance was almost completely restored. Load drivers will serve as fundamental building blocks in the creation of complex, higher-level genetic circuits.

Understanding the limits of modularity in biological systems and developing appropriate mechanisms to overcome these limitations is an important challenge in the design and construction of synthetic systems6–8. Modularity can fail at different levels of the system hierarchy, for example, interference between promoter and transcript regions due to structural interactions between DNA and RNA or proteins or functional interactions with host factors and metabolites9–11. At the level of genetic parts, it was shown recently that promoter–transcript interference can be addressed by cleaving a transcript through the addition of so-called insulators such as ribozymes6 or clustered regularly interspaced short palindromic repeat (CRISPR)-mediated cleavage7. However, modularity failures at the level of circuit topology have not yet been addressed. At this level, protein signals from devices or modules such as an oscillator10,11, a toggle switch12 or an activation cascade13 can serve as input and output signals to perform human-defined regulatory functions14,15. For building complex multi-module circuits, it is desirable that module behavior (as characterized individually) does not change substantially when creating functional connections to other modules.

One common method for engineering a connection between an upstream and a downstream module in transcriptional networks is to have the upstream module’s output protein bind DNA operator sites in promoters of the downstream module. Analogously, modules in engineered protein networks can be connected through protein docking domains. Reversible binding reactions between upstream regulatory proteins and downstream binding sites (for example, DNA operators or protein docking domains) create load that can temporarily sequester the regulatory proteins from other reactions, resulting in undesirable delays or disruptions in system function. Loads have substantial effects on system behavior, and these effects are known as retroactivity5,15–19.

Retroactivity has been detected in both natural and synthetic biological systems. In the endogenous Drosophila MAPK pathway, phosphorylation levels of the upstream Ras-ERK module are perturbed by concentration changes of the downstream substrates Cic and Gro, resulting in retroactive effects that contribute to spatial regulation of early embryonic gene expression15,20. Experiments on a reconstituted PII-NRII signal transduction cascade of Escherichia coli in a cell-free environment demonstrated that downstream NRII targets markedly affect the upstream (UTase/UR)-PII cycle’s temporal response16. Although natural systems encode network topologies that function despite retroactivity, or may sometimes even exploit it15,20, design of synthetic networks is often confounded by retroactivity. Experiments with synthetic networks in E. coli have validated the undesirable impact of retroactivity, such as in a transcriptional repression cascade whose temporal response is substantially affected by addition of a downstream module encoding transcription factor target operators17. Another experiment in E. coli showed that the steady-state input-output characteristic of an upstream repressor module changes substantially when a downstream system with the repressor’s binding sites is added18. Therefore, creation of large-scale synthetic transcriptional networks will be difficult without design strategies that overcome problems of modular composition.

To mitigate retroactivity, we report the design and implementation of a load driver, a fast phosphotransfer-based device that is placed between slower upstream and downstream transcriptional modules (Fig. 1a,b). Incorporation of fast processes as a bridge between slower processes exemplifies the design principle of timescale separation to insulate an upstream module from load applied by its downstream module21. We obtained the design principle of the load driver by mathematically formulating the issue of load as a control theoretic
problem of disturbance attenuation\(^1\) (Supplementary Note 1.1 and 1.2). We provide a simplified analysis of how separation of timescale is used to attenuate retroactivity (Box 1 and Fig. 2). By virtue of its fast dynamics, the load driver responds almost instantaneously to the slower temporal changes in its input and quickly reaches a quasi-steady state (QSS), such that the comparatively slower changing input seems constant. Load from the downstream module is transferred to the load driver's output and can affect both the time needed to reach the quasi-steady state (QSS) and the downstream system's ability to attenuate retroactivity. The combined effect is that the load driver mitigates retroactivity, and the operations of the upstream and downstream modules are independent of their connectivity.

To experimentally characterize the load driver's performance in attenuating retroactivity, we designed and integrated four system types (Fig. 1c–e) into S. cerevisiae. All four systems have identically functioning upstream modules with doxycycline (DOX) as input and an output module containing GFP as output. The systems differ in whether they include a load module and a load driver. The upstream modules contain constitutively expressed reverse tetracycline transactivator protein (rtTA), which induces PTET promoter expression in the presence of DOX. Unbuffered systems do not include the load driver, and the upstream module is connected directly to the output module and, if present, the load module (Fig. 1d). In the buffered systems, we introduced an intervening load driver module that incorporates a phosphotransfer cascade between the upstream and output modules (Fig. 1e). Comparison of circuit behaviors with and without the load module in both systems allowed us to determine the load driver's ability to attenuate retroactivity.

In the unbuffered system (Fig. 1d and Supplementary Fig. 1), PTET regulates expression of SKN7m, a constitutively active phosphorylated mutant of the nuclear aspartate response regulator SKN7 (ref. 22). SKN7m activates expression of GFP from the synthetic promoter PTET-SSRE (ref. 23) to provide an output readout in response to DOX input. All system elements are chromosomally integrated except for the load variants, which are encoded on high-copy 2µ plasmids. Experiments were performed in S. cerevisiae strain with a hot1∆ho1Δssn1Δ background (Online Methods).
Box 1 Timescale separation for retroactivity attenuation

Here we provide a simplified mathematical explanation of how retroactivity can be attenuated by a load driver that utilizes processes with timescales that are much faster than those of its flanking modules. A more in-depth and general mathematical analysis appears in Supplementary Note 1.2. Consider the block diagram in Figure 1b, in which proteins create functional connections from the load driver to the upstream and downstream transcriptional systems. We define $u(t)$ as the load driver’s time-varying input protein concentration, $y$ as the concentration of the load driver’s output protein in its free active form, and $c$ as the concentration of load driver’s output protein bound to DNA binding sites in the downstream system.

To illustrate how timescale separation results in attenuation of retroactivity, we consider a basic model of the isolated load driver encoding processes that generate and remove the output protein $y$. We define the lumped parameter $G$ to scale together the rates of production and removal of $y$ yielding $G \cdot (u(t) - y)$. Here, larger values of $G$ correspond to faster timescales of load driver dynamics. After interconnection with load, other reactions that affect $y$ include reversible binding to downstream DNA sites in concentration $p$ with the rate $k_{on} \cdot p \cdot y - k_{off} \cdot c$, in which $k_{on}$ and $k_{off}$ are ‘on’ and ‘off’ binding rate constants, respectively. The resulting system dynamics can be represented by two differential equations:

$$\frac{dy}{dt} = G \cdot (u(t) - y) - k_{on} \cdot p \cdot y + k_{off} \cdot c \quad (1)$$

$$\frac{dc}{dt} = k_{on} \cdot p \cdot y - k_{off} \cdot c$$

The reversible binding reactions between the load driver’s active output protein and downstream DNA binding sites constitute retroactivity $r$, that is, $r = k_{on} \cdot p \cdot y - k_{off} \cdot c$, and is shown in red. We refer to the system with $r = 0$ as the unloaded system and to the system where $r$ is nonzero as the loaded system.

We seek to understand how the time-dependent response of $y$ to $u(t)$ is affected by retroactivity when the timescale of the load driver dynamics becomes faster ($G$ increases) in comparison to the speed of the input. To this end, consider a particular example where $u(t)$ is a periodic input (Fig. 2a). The simulation in Figure 2b shows the system response to this input (red) or unloaded (black) system when the load driver operates at a slow timescale (low $G$). At slow timescales, the loaded system is unable to respond to the input signal effectively. In comparison, Figure 2c shows a simulation for the same periodic input $u(t)$ with a load driver operating at a fast timescale (high $G$), where the effect of retroactivity is attenuated.

To understand this phenomenon, it is useful to graph the ratio between the oscillatory amplitudes of the output and the input as a function of the input frequency $\omega$, that is, the magnitude $M$ of the system’s frequency response gain. This is a common way to determine how a system responds to its input when the relative timescales change. Figure 2d shows $M$ as a function of the input frequency $\omega$ for the systems with low $G$ (Fig. 2b). Figure 2e shows the corresponding relationship for the systems with high $G$ (Fig. 2c), as derived in Supplementary Note 1.2,

$$M(\omega) = \frac{\alpha \cdot G}{\sqrt{\omega^2 + (\alpha \cdot G)^2}},$$

in which $\alpha = 1$ for the unloaded system and $\alpha = (1 + \frac{p}{K_d})^{-1}$ for the loaded system, where $K_d = k_{off}/k_{on}$ is the dissociation constant of the binding reaction.

The cutoff frequency (also called bandwidth) is a convenient metric for quantifying the speed of a system and is defined as the frequency of the input such that the magnitude $M$ drops below $1/\sqrt{2}$ (ref. 35). This frequency can be found by solving the equation $M(\omega) = 1/\sqrt{2}$ for $\omega$, and for system (1) is equal to $\alpha \cdot G$. Hence, when $\omega \ll \alpha \cdot G$, that is, the rate of change of the input is much slower than the dynamics of the load driver (shaded areas in Fig. 2d,e), we have that $M(\omega) = 1$. This implies that $y(t) = u(t)$, which also corresponds to the QSS value of the output obtained by viewing $u(t)$ as a constant input in system (1) and by solving for the steady state. In this case, the output $y$ is able to effectively follow $u(t)$ independent of retroactivity. As retroactivity reduces the cutoff frequency since $\alpha < 1$ (compare the black and red plots in Fig. 2d), an increase in $G$ for the load driver extends the range of input frequencies where retroactivity is attenuated, i.e., where $M(\omega) = 1$ (compare the red plots in Fig. 2d,e). Considering the input signal $u(t)$ given in Figure 2a, the magnitude of the frequency response gain of the unloaded and loaded systems corresponding to this specific input are marked by X and +, respectively, within Figure 2d,e. When the separation of timescale between the load driver’s dynamics and the input is increased, i.e., $G$ is increased, the difference in $M$ is eliminated, and thus retroactivity is attenuated.

Figure 2 Attenuation of retroactivity by faster load driver dynamics. (a) An example periodic input $u(t)$. (b,c) Simulated system responses with a slow timescale (low $G$) and a fast timescale (high $G$), respectively, to the input in a. (d,e) Magnitude $M$ as a function of the input frequency $\omega$ for a system with a slow timescale (low $G$) and a fast timescale (high $G$), respectively. In d and e, the shaded region represents the range of frequencies at which retroactivity has a negligible effect, and this range increases with higher $G$. The bandwidths of the unloaded systems are marked by dashed lines. The magnitudes of the oscillatory system outputs from b and c (input frequency in a) are indicated by + and X, respectively, in e.
plasmids (50–100 copies per cell)\textsuperscript{24}, each with zero (unloaded), one (single loaded) or two (double loaded) additional copies of P\textsubscript{TR-SSRE} (Supplementary Figs. 1 and 2). The load creates reversible binding reactions between SKN7\textsubscript{m} and the corresponding operators within P\textsubscript{TR-SSRE}, resulting in additional flux (retroactivity) that affects the rate of change of SKN7\textsubscript{m} available to activate the output\textsuperscript{5,17}. For the unbuffered circuits, after induction with DOX, the total amount of SKN7\textsubscript{m} increases with time, but a portion binds P\textsubscript{TR-SSRE} operators, slowing down the increase of free SKN7\textsubscript{m} available to activate system output.

In the buffered systems (Fig. 1e and Supplementary Fig. 1), DOX binds rtTA and induces expression of a new fusion protein, STAT5-HKRR, comprising mouse signal transducer and activator of transcription 5 (STAT5) fused to the histidine kinase (HK) and response regulator (RR) domains of the yeast synthetic lethal to N-end rule protein (SLN1). This protein (described in the Online Methods) serves as input to the load driver. Within the load driver, a protein that consists of the JH1 domain from mouse Janus kinase 2 (JAK2) is constitutively expressed. This JH1 domain autophosphorylates before phosphorylating the STAT5 domain of STAT5-HKRR, resulting in dimerization of STAT5-HKRR\textsuperscript{15}. STAT5-mediated dimerization enables HK autophosphorylation, phosphorylation to the RR domain and subsequent phosphotransfer to constitutively expressed tyrosine phosphate–dependent protein (YPD1). After activation, two phosphorylated YPD1 (pYPD1) proteins reversibly transfer their phosphates to constitutively expressed SKN7 to form doubly phosphorylated SKN7 (ppSKN7; refs. 22,26). ppSKN7 activates expression of GFP from chromosomally integrated P\textsubscript{TR-SSRE} and also binds plasmid-encoded load sites. Whereas the input module's output protein (SKN7\textsubscript{m}) in the unloaded systems binds P\textsubscript{TR-SSRE} directly, the corresponding protein (SKN7) in the buffered system's load driver module requires activation by a sequence of phosphotransfer reactions before binding P\textsubscript{TR-SSRE}.

To analyze the overall design and components crucial for the operation of our load driver, we developed a detailed mathematical model based on mass-action kinetics and ordinary differential equations (ODEs) (Supplementary Note 1.3 and Supplementary Fig. 3). This analysis showed that sufficiently large phosphotransfer rates ensure the timescale separation required for quickly reaching the QSS, and sufficiently high concentrations of SKN7 and YPD1 render this QSS independent of load (Supplementary Note 1.4–1.6). Specifically, large amounts of these proteins guarantee that the steady-state sequestration of ppSKN7 by the promoter binding sites can be effectively compensated by activation of additional SKN7 (Supplementary Note 1.6.4). We chose to make two versions of the buffered system (Fig. 1e), with one version encoding promoters expressing SKN7 and YPD1 at relatively low levels and a second version encoding promoters expressing these proteins at moderate wild-type levels. This and several additional design choices in the creation of our experimental systems are described in the Online Methods.

For our initial experimental characterization, we conducted time-series experiments with a single step input change applied to all systems. We introduced DOX to log-phase liquid cultures without a prior inducer (step up) or removed DOX from cultures previously grown in saturating inducer (step down). For the step-up experiment, the loaded unbuffered system exhibited 19.8 ± 2.7% (2× load) (1× load) and 30.7 ± 2.7% (2× load) (Fig. 3a and Supplementary Fig. 4). Although the unbuffered system eventually reached the same levels of expression regardless of load, the final output levels of the buffered system with low SKN7 and YPD1 expression were reduced as a result of load (Fig. 3b and Supplementary Figs. 4 and 5). In contrast, the loaded buffered system with moderate SKN7 and YPD1 expression was unaffected by load, in both its temporal response and final output (Fig. 3c). Because the buffered system with moderate expression levels of SKN7 and YPD1 was able to attenuate retroactivity, we chose it for further evaluation. For this system, we observed identical behavior when the load module included fluorescent reporters downstream of P\textsubscript{TR-SSRE} (Supplementary Fig. 6). Simulations performed with a
detailed model of these biochemical processes correlated well with the theoretical analysis and experimental observations (Fig. 3 (insets) and Supplementary Fig. 7). Of note, although one might expect that addition of regulatory elements to a critical path of a system would slow down response times, here we observed the converse for the buffered compared to the unbuffered loaded systems.

We also examined the steady-state behavior of the systems. We performed step-up time-series experiments with intermediate load plasmids and grown in liquid culture (OD660 maintained at 0.2–0.6 by periodic dilution). Gray shading indicates application of 20 μM DOX. The error bars in c indicate the s.d. for n = 3 oscillations of an individual trajectory. (a) Experimental GFP response trajectories from single cultures for unbuffered (left) and buffered (right) circuits when periodically induced by 20 μM DOX followed by removal after 50 min. Injection time corresponds to the time between successive DOX inductions. (b) Simulated GFP trajectories from a mathematical model using a parameter set fitted to full data sets with periodic DOX induction as described in a. The parameters used for the simulations are given in Supplementary Table 1. (c) Amplitude of the responses of unbuffered (left) and buffered (right) systems across injection frequencies. Dotted lines correspond to calculated bandwidths, and for the unbuffered system these values are 0.0140 min−1 and 0.0175 min−1 for the unloaded and loaded conditions, respectively. The buffered system has bandwidths of 0.0142 min−1 and 0.0143 min−1 for the unloaded and loaded conditions, respectively.

Figure 4  System responses to periodic inputs. (a–c) S. cerevisiae cells integrated with circuits with 0x (black) or 2x (red) load plasmids and grown in liquid culture (OD660 maintained at 0.2–0.6 by periodic dilution). Gray shading indicates application of 20 μM DOX. The error bars in c indicate the s.d. for n = 3 oscillations of an individual trajectory. (a) Experimental GFP response trajectories from single cultures for unbuffered (left) and buffered (right) circuits when periodically induced by 20 μM DOX followed by removal after 50 min. Injection time corresponds to the time between successive DOX inductions. (b) Simulated GFP trajectories from a mathematical model using a parameter set fitted to full data sets with periodic DOX induction as described in a. The parameters used for the simulations are given in Supplementary Table 1. (c) Amplitude of the responses of unbuffered (left) and buffered (right) systems across injection frequencies. Dotted lines correspond to calculated bandwidths, and for the unbuffered system these values are 0.0140 min−1 and 0.0175 min−1 for the unloaded and loaded conditions, respectively. The buffered system has bandwidths of 0.0142 min−1 and 0.0143 min−1 for the unloaded and loaded conditions, respectively.
DOX levels (Supplementary Fig. 8) and used these to generate the steady-state dosage response of our systems. In agreement with our models, neither the inclusion of the load driver nor the presence of load affected the final steady state (Fig. 3d–f). For the unbuffered system, the fact that the steady state did not change with the addition of load is consistent with a system in which the interconnecting species (SKN7m) is protected from degradation when bound to DNA17. This observation is in contrast to those from other studies27,28 where interconnecting species are not protected from degradation when bound to DNA, and load may result in steady-state effects such as ultrasensitivity and thresholding. For the buffered system, the steady state did not change with the load, provided that the amounts of SKN7 were sufficiently high (Fig. 3e,f). Our theoretical analysis showed that the load driver steady-state dosage response is essentially linear and is determined by the ratio between the spontaneous rates of STAT5-HKRR phosphorylation and ppSKN7 dephosphorylation (Supplementary Note 1.5). Because the response curve slope is relatively insensitive to all other parameters, when these two reaction rates are well balanced, the slope is approximately equal to one, and hence the load driver does not affect the steady state (Supplementary Note 1.6 and Supplementary Figs. 9 and 10).

To further characterize the load driver’s ability to attenuate retroactivity, we next considered time-varying inputs. Specifically, we assayed system responses to periodic square wave inputs. We used the simulation model shown in Figure 3 to suggest input waveforms that display large retroactivity effects (Supplementary Note 1.3.3 and Supplementary Figs. 11 and 12). We initially applied saturating DOX, followed by periodic square wave inductions with a fixed on...
time of 50 min and varying periods (150, 200, 250, 350 and 500 min) to exponentially growing cultures. We measured system trajectories by flow cytometry (Fig. 4a and Supplementary Fig. 13), and the experimental results correlated well with the simulations (Fig. 4b). For the waveforms analyzed, load on the unbuffered system resulted in an ~50-min phase lag, a 44–81% decrease in the peak-to-peak amplitude of oscillations and a 25% bandwidth reduction (Fig. 4c). By contrast, the buffered system exhibited almost no lag in the phase responses, minor amplitude deviations (4–12%) and negligible (0.5%) changes in bandwidth due to load (Fig. 4c). Dynamic sensitivity analysis yielded parameter conclusions that were similar to those obtained in steady-state conditions (Supplementary Figs. 14–16).

In summary, load drivers can mitigate retroactivity when they operate at timescales much faster than those of the flanking modules, and their QSS is independent of load. In our systems, the flanking modules are transcriptional networks with timescales in minutes to hours, as determined by gene expression and protein decay.29 Hence, molecular mechanisms with timescales in the seconds or subseconds are good candidates for a load driver, including phosphorylation, phosphotransfer and methylation. To achieve the requirement of timescale separation, we chose to use a multistage phosphotransfer cascade (STAT5-HKRR to YPD1 to SKN7 phosphotransfer)22,25 to implement our load driver. The amount of YPD1 and SKN7 used ensured the fast timescale essential for quickly approaching the QSS and negligible retroactivity effects on the QSS itself. In the current experimental setup, YPD1 and SKN7 levels were moderate and were estimated to be at the 75th and 53rd percentiles (6,325 and 2,572 protein copies per cell, respectively) of yeast protein expression levels.30 For load drivers to handle increasing amounts of load at the same level of performance requires an approximately linear increase in the concentrations of YPD1 and SKN7, up to some limit (Supplementary Figs. 17 and 18), which can be accomplished readily with stronger promoters. Furthermore, the load driver’s output QSS should be an approximately linear function of the input. Criteria for obtaining this linear characteristic depend on the molecular mechanisms and have been studied extensively for covalent modification processes.31,32 We chose to implement the load driver using a multistage phosphotransfer cascade, as opposed to (for example) a one-stage cascade. With one-stage cascades, the requirement that the QSS is independent of load is in conflict with the requirement of fast load driver dynamics,33 but this is not a limitation for systems with multiple stages (as described in detail in Supplementary Note 1.7 and Supplementary Figs. 19–21).

So far, the creation of synthetic gene networks has focused largely on engineering circuits that involve only slow processes, such as gene expression, or only fast processes, such as signal transduction. By contrast, in this manuscript we describe construction of a system based on a new design principle for engineering biological systems that combines slow and fast processes to mitigate loading effects in connected modules. We anticipate that in synthetic biology, load drivers will serve a role similar to that of unity-gain amplifying buffers in electronics. These buffers enable reliable and predictable connection of subsystems by eliminating load-induced alterations to the input-output characteristics of these subsystems.34 In electronics, selective incorporation of amplifying buffers simplifies and substantially speeds up the design process, as circuits no longer require optimization or even redesign when new connections are formed. Analogously, we envision that selective incorporation of multiple orthogonal load drivers into synthetic gene circuits will mitigate retroactivity arising from module interconnections (Box 2, Fig. 5). Generation of load driver libraries and effective rules for incorporating load drivers into synthetic circuits will foster more predictable creation of complex systems.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** Sequences are available in GenBank for pLDUNB (KM457489), pLDUF (KM457486), pLDUBFL (KM457486), pLD00X (KM457490), pLD01X (KM457485) and pLD02X (KM457487).

**Note.** Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

D.M., D.D.V. and R.W. designed the experiments and analyzed the data. D.M. performed the experiments. P.M.R. constructed mathematical models and performed parameter estimation. A.L. cloned constructs. D.M., D.D.V. and R.W. wrote the paper.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Experimental reagents. Nucleic acid manipulation. AccuPrime Pfx SuperMix from Life Technologies and oligonucleotides manufactured by Integrated DNA Technologies were used for all PCR amplification. The oligonucleotides used for PCR are listed in Supplementary Table 2, those used for sequencing are listed in Supplementary Table 3, and those used to generate yeast knock-outs are listed in Supplementary Table 4. BP Clonase II Enzyme Mix and LR Clonase II Plus Enzyme Mix from Life Technologies were used for all BP and LR reactions, respectively (Supplementary Table 5). Taq ligase, NAD+, dNTPs and Phusion Polymerase from New England Biolabs and T5 Exonuclease from Epicentre Biotechnologies were used for Gibson assembly reactions to form the plasmids listed in Supplementary Table 6 (ref. 44). All other restriction enzymes were obtained from New England Biolabs. All buffer components were obtained from Sigma-Aldrich.

Bacteria. All plasmid constructions listed in Supplementary Tables 5 and 6 used E. coli strain E. coli strain 10G (Lucigen) made chemically competent by a kit (Zymo Research) and transformed by the suggested Z-comp heat-shock procedure. Bacterial cultures were grown in Luria-Bertani medium (BD Biosciences) and supplemented with kanamycin, ampicillin and chloramphenicol (Sigma-Aldrich), as appropriate.

Yeast. All strains used are listed in Supplementary Table 7 and were prepared using standard transformation procedures in a yeast strain with a W303 background, YSC108 (ThermoFisher Scientific). 5-fluoroorotic acid (5-FOA) was bought from Zymo Research, phleomycin was from Invivogen, and G418 was from Stratagene. All synthetic defined URA (SD-URA) dropout (5-FOA) was bought from Zymo Research, phleomycin was from Invivogen, and G418 was from Stratagene. All synthetic defined URA (SD-URA) dropout (5-FOA) was bought from Zymo Research, phleomycin was from Invivogen, and G418 was from Stratagene.

DNA circuit construction. The hierarchical assembly protocol outlined in Guye et al. was followed using yeast-specific components.

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We chose to knock out HOG1 and HOT1 instead of further knocking out the high-osmolarity pathway component SKN1 or SSK2 because ΔSSK1 would have exacerbated lethality and ΔSSK2 would not have guaranteed rescue as a result of SSK2 redundancy and/or PBS2 promiscuity, leading to possible HOG1 activation. The deletion strain (ΔHOG1ΔHOT1ΔSLN1) growth characteristics were similar to those of wild-type strains under normal growth conditions (Supplementary Fig. 22).

In the case of the buffered system with low YPD1 and SKN7 expression, a second deletion strain was desired (YL22) with the phenotype ΔHOG1ΔHOT1ΔSLN1 background). However, because the YPD1 deletion is synthetic lethal, this strain was constructed by first knocking out SKN7 then subsequent integration of plBDUFL into the HO locus and then knocking out YPD1 as described below. The promoter pairs for SKN7 and YPD1 expression were chosen from the literature to express at approximately 10–30% of wild-type SKN7 and YPD1 levels.53,54

We integrated all exogenous transcriptional units as a single copy with kanMX selection into the growth-neutral HO locus and did not experimentally observe any alterations to the growth characteristics (Supplementary Fig. 18). To maintain promoter activity as our device output interface, the load must consist minimally of identical PTR-SSRE promoter sequences. These promoters were fused with downstream terminator sequences (Supplementary Fig. 2). All transformations used the standard LiAc protocol.55 A list of all oligonucleotides used in the gene disruption is shown in Supplementary Table 4, and a summary of all yeast strains appears in Supplementary Table 7.

A special multi-reporter load plasmid was constructed using the LR-Gibson assembly method described below with the first TR-SSRE promoter driving mCherry and the second TR-SSRE promoter driving azurite. The experimental results are reported in Supplementary Fig. 6.

Yeast strain with gene deletions. All knockout strains were prepared by Cre-lox genetic disruption as described previously.55 YLD (W303A in ΔHOG1ΔHOT1ΔSLN1 background): (i) intermediate W303 in the ΔHOG1 background: loxp-flanked K. lactis URA3 was amplified by PCR from pUG72 (ref. 55) (EUROSCARF accession number P30117) using LDk001 and LDk002 and then transformed into W303 yeast before selection. Clones were verified for successful integration by colony PCR before following standard transformation of the Cre-containing plasmid, pSH65 (ref. 55) (EUROSCARF accession number P30122), followed by selection and counterselection protocols and colony PCR verification. (ii) Intermediate W303 in the ΔHOG1ΔHOT1 background: loxp-flanked K. lactis URA3 was amplified by PCR from pUG72 using LDk003 and LDk004, and then that product was amplified further by LDk005 and LDk006. This PCR fragment was transformed into the W303 strain with the ΔHOG1 background described above. Subsequent steps proceeded as described in the HOG1 deletion procedure. (iii) Final strain, YLD: loxp-flanked K. lactis URA3 was amplified from pUG72 using LDk007 and LDk008. This PCR fragment was transformed into the W303 with the ΔHOG1 ΔHOT1 background described above. Subsequent steps proceeded as described previously in the HO1 deletion procedure.

YLD2 (W303 in the ΔHOG1ΔHOT1ΔSLN1ΔASKN7ΔYPD1 background): (i) intermediate W303A in the ΔHOG1ΔHOT1ΔSLN1ΔASKN7: loxp-flanked K. lactis URA3 was amplified by PCR from pUG72 using LDk009 and LDk010. This PCR fragment was transformed into the YLD strain described above. Subsequent steps proceeded as described in the HO1 deletion procedure. (ii) Intermediate W303A in ΔHOG1ΔHOT1ΔSLN1ΔASKN7 with plBDUFL: integration of plBDUFL proceeded as described below. (iii) Final strain YLD2: loxp-flanked K. lactis URA3 was amplified from pUG72 using LDk011 and LDk012. This PCR fragment was transformed into the strain described above. Subsequent steps proceeded as described previously in the HOG1 deletion procedure. The strains all had similar growth characteristics to wild-type strains (Supplementary Fig. 22).

Yeast strains with integrated circuits and load. All circuit strains with load were prepared in the following manner. First, the unbuffered and shifted buffer system plasmids (pLDUNB, pLDUFL and pLDUF) were linearized with ScaI and purified by the Qiagen PCR Cleanup Kit before subsequent LIAc transformation into strain YLD and selection on YPAD solid medium supplemented with URA and G418. This step was repeated for the buffered clone, yielding a total of nine strains, YLDU00, YLDU01, YLDU02, YLDBL00, YLDBL01, YLDBL02, YLD000, YLD001 and YLD002, with the properties listed in Supplementary Table 7. These strains follow the naming convention yeast load driver (YLD) followed by U, B or BL for integrated unbuffered, buffered or buffered low system, respectively, and ending with 00, 01 or 02, corresponding to the amount of load.

Cell culture. Yeast strains harboring circuits and load, YLDU00, YLDU01, YLDU02, YLD000, YLD001 and YLD002, were grown in SD-URA liquid medium supplemented with G418 for 16 h and combined with equal parts glycerol to form cell stocks. For all experiments, these cell stocks were used to inoculate 4-mL liquid cultures and were grown under full selection for 12 h at 30 °C on a rotary shaker at 280 r.p.m. These ‘starter cultures’ were diluted to OD_{660} = 0.10 and grown under selection for 2 additional hours at 30 °C with 280 r.p.m. rotary shaking before being collected by centrifugation and resuspended in fresh selection medium for experiments. OD_{660} was obtained using a NanoDrop 2000c (NanoDrop, Wilmington, DE) and calibrated to cell density counts using a hemocytometer calibration curve (Supplementary Fig. 22).

Step-up perturbation. Cultures at OD_{660} = 0.20 were grown under selection at 30 °C on a rotary shaker at 280 r.p.m. in 4-mL liquid cultures for 1,060 min with aliquots removed every 20 min (t = 0–300 min) and every 40 min (t = 300–1,060 min) for cytometry. 20 μM DOX was added to the liquid cultures at t = 100 min and maintained for the full 1,060 min. OD_{660} was maintained between 0.20 and 0.60 using periodic dilution of cultures with fresh medium containing the appropriate inducer.

Step-down perturbation. Cultures at OD_{660} = 0.20 were grown under selection at 30 °C on a rotary shaker at 280 r.p.m. in 4-mL liquid cultures for 1,000 min with 20 μM DOX and periodic dilution with fresh medium containing the appropriate inducer to keep OD_{660} between 0.20 and 0.60. After 1,000 min, cultures were diluted to OD_{660} = 0.2 with DOX at t = 0 min and grown in 4-mL liquid cultures with aliquots drawn at 50 and 100 min. At 100 min, cultures were centrifuged to remove DOX and grown under selection in 4-mL liquid cultures for 1,800 min, with aliquots removed every 50 min for cytometry. OD_{660} was maintained between 0.20 and 0.60 using periodic dilution of cultures with fresh medium.

Periodic injections. Cultures at OD_{660} = 0.20 were grown under selection at 30 °C on a rotary shaker at 280 r.p.m. in 4-mL liquid cultures for 1,500 min with DOX introduction or removal by centrifugation and periodic dilution with fresh medium to maintain OD_{660} between 0.20 and 0.60. Aliquots were removed every 50 min for cytometry. The initial induction times were as follows: 150 min injection, 150 min induction; 200 min injection, 150 min induction; 250 min injection, 125 min induction; 350 min injection, 50 min induction; and 500 min injection, 50 min induction. After these induction times, wave forms with 50-min high DOX and the remainder of the injection period absent DOX were applied.

Flow cytometry measurement. The same LSRFortessa flow analyzer (BD Biosciences) was used for all flow cytometry measures using the following settings. eGFP was measured using a 488-nm laser and a 530/15 emission filter using a photomultiplier tube (PMT) setting of 360 V. For each sample, 30,000 events were collected and gated according to forward scatter (FSC-A) (PMT of 130 V) and side scatter (SSC-A) (PMT of 100 V). In parallel, Rainbow Calibration Particles (Spherotech RCP-30-5A) were measured to equalize data between different experimental runs (as described in the next section).

Data analysis. Flow cytometry. The line plots in Figure 3 and Supplementary Figures 4 and 5 were generated as follows. For each sample, the corresponding reference standard calibration was defined using FlowJo (TreeStar Software) to obtain a new channel, called FITC-Calibrated was calculated for each sample. Those median values were then graphed (or the mean of replicate medians, in the case of Figure 3) using Prism (GraphPad Software). Adjacent points are connected by straight lines.

Rise and decay times. Rise times for the step-up input in Figure 3 were calculated for only the unbuffered and moderate buffered systems as follows. Eighteen individual data sets (three replicates of unbuffered 0x, unbuffered 1x, unbuffered 2x, moderate buffered 0x, moderate buffered 1x and moderate buffered 2x) were loaded into MATLAB (The Mathworks, Natick, MA).

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For each individual trajectory, a minimum value, the average of the first three data points (t ≤ 0 min) for each single trajectory, was calculated. The minimum value was subtracted from every data point of its corresponding trajectory to eliminate bias, i.e., the data range was shifted to start at zero rather than ~300 AU from the instrument. For the three unbuffered 0x trajectories and three buffered 0x trajectories, a maximum value, the average of the last three data points of each single trajectory, was calculated. These individual 0x trajectories were averaged to yield UnbufferedMaxMean and BufferedMaxMean. All nine unbuffered trajectories were normalized by dividing each data point by UnbufferedMaxMean and all nine buffered trajectories were normalized by dividing each data point by BufferedMaxMean. This normalization allows for accurate comparison between loaded systems and the control, unloaded system. For each unbuffered trajectory, linear piecewise interpolation was performed to obtain the response time, or the time necessary to reach 90% of UnbufferedMaxMean. This procedure was repeated for buffered trajectories using BufferedMaxMean. The interpolated rise times were averaged, and the means and s.d. values are as follows: unbuffered: 0x load, 396.1 ± 23.0 min; 1x load, 474.6 ± 45.3 min; 2x load, 698.9 ± 47.9 min; buffered: 0x load, 432.4 ± 8.9 min; 1x load, 420.2 ± 20.9 min; and 2x load – 423.2 ± 21.5 min. To calculate the slow down to reach 90% of the maximum value as reported in the main text, the following formula was employed:

\[
\text{Error in percentages} = \frac{\text{LoadedMean} - \text{UnloadedMean}}{\text{UnloadedMean}} \times 100\%
\]

Decay times for the step-down input in Figure 3 were calculated similarly to the rise times for only the unbuffered and moderate buffered system as follows with several deviations. For each individual trajectory, a minimum value, the average of the last three data points for each single trajectory, was calculated. This minimum value was subtracted from every data point of its corresponding trajectory to eliminate bias, i.e., the data range was shifted to start at zero rather than ~300 AU from the instrument. For the three unbuffered 0x trajectories and three buffered 0x trajectories, a maximum value, the average of the last three data points (t ≤ 0 min) of each single trajectory, was calculated. These individual 0x trajectories were averaged to yield UnbufferedMaxMean and BufferedMaxMean. The trajectories were normalized to their corresponding average. For each unbuffered trajectory, linear piecewise interpolation was performed to obtain the response time, or the time necessary to reach 10% of UnbufferedMaxMean. This was repeated for buffered trajectories using BufferedMaxMean. The interpolated decay times were averaged, and the means and s.d. values are as follows: unbuffered: 0x load, 1,044.1 ± 31.2 min; 1x load, 1,256.6 ± 19.4 min; 2x load, 1,364.6 ± 12.9 min; buffered: 0x load, 1,024.4 ± 36.6 min; 1x load, 1,021.9 ± 38.5 min; and 2x load, 1,043.6 ± 23.6 min. To calculate the slow down to reach the 10% maximum value as reported in the main text, the means and deviations were used in the same formulas as shown above.

The slow down for the unbuffered systems are reported in the main text. For the buffered system with moderate SKN7 and YPD1 expression, their values are reported here. The step-up response exhibited 2.83 ± 2.84% (1x load) and 2.13 ± 2.94% (2x load) changes in rise time compared to the unloaded buffered system, and for the step-down experiment, the system had 0.24 ± 0.25% (1x load) and 1.88 ± 1.27% (2x load) changes in decay times.

Amplitude attenuation. The amplitudes shown in Figure 4b were calculated as follows. The data sets were loaded into MATLAB, and the local minimum and local maximum values of trajectories starting at the injection point until the next injection point were found. The corresponding values were subtracted such that a peak-to-peak amplitude was obtained. The averages and s.d. values plotted were obtained using the last three injections of each 1,500-min experiment. System amplitude differences expressed as the relative error between the loaded and unloaded conditions are as follows: unloaded: 150 min, 44.1 ± 25.8%; 200 min, 80.8 ± 3.8%; 250 min, 61.8 ± 0.9%; 350 min, 49.2 ± 2.0%; 500 min, 57.6 ± 2.2%; buffered: 150 min, 5.9 ± 10.7%; 200 min, 7.6 ± 25.6%; 250 min, 12.2 ± 5.3%; 350 min, 4.0 ± 4.5%; and 500 min, 5.2 ± 3.7%.

Mathematical analyses. We include a Supplementary Note that contains several sections and additional figures providing mathematical and computational methods and analyses in support of the load driver. Supplementary Note 1.1 describes the use of timescale separation for retroactivity attenuation, and Supplementary Note 1.2 contains the mathematical derivations necessary for Box 1. Supplementary Note 1.3–1.5 provides a comprehensive description of the formulation, assumptions and parameters for the circuits shown in Figure 1. Supplementary Note 1.4–1.6 includes mathematical analyses to analyze the retroactivity attenuation property (Supplementary Fig. 23), assess parameter sensitivity of the load driver (Supplementary Figs. 9, 10 and 14–16) and calculate error due to retroactivity (Supplementary Figs. 17 and 18). In addition, Supplementary Note 1.7 and 1.8 includes mathematical analyses and results of load drivers built with one or two stages (Supplementary Figs. 19–21), as well as the use of load drivers and load encoded on medium-copy plasmids instead of high-copy or integrated circuits (Supplementary Fig. 24).

Unbuffered and buffered models summary. We formulated mathematical models for the unbuffered and buffered circuits shown in Figure 1. We used these models to both assess the effect of retroactivity in the specific circuit implementations chosen for the experiments and demonstrate that the chosen load driver circuit implementation in Figure 1e satisfies the structure and assumptions required for the retroactivity attenuation described in Supplementary Note 1.1. Each system was written as a set of reactions governing protein species and then using mass-action kinetics, formed into a set of ODEs. These ODEs were then used to construct a gray-box model using the MATLAB System ID Toolbox (The Mathworks). Experimental data sets were used to fit parameters of the gray-box model using the trust-region-reflective least-squares algorithm. Final simulations were performed using a stiff differential equation solver (MATLAB ode23s) and the final parameter values listed in Supplementary Table 1. A comprehensive description of the model is included in the Supplementary Note. A mapping between the mathematical model and the schematics from the main text appears in Supplementary Note 1.3. Additionally, the code used is available online as Supplementary Code.

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