Design Principles for Regulator Gene Expression in a Repressible Gene Circuit

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Summary

We consider the design of a type of repressible gene circuit that is common in bacteria. In this type of circuit, a regulator protein acts to coordinately repress the expression of effector genes when a signal molecule with which it interacts is present. The regulator protein can also independently influence the expression of its own gene, such that regulator gene expression is repressible (like effector genes), constitutive, or inducible. Thus, a signal-directed change in the activity of the regulator protein can result in one of three patterns of coupled regulator and effector gene expression: direct coupling, in which regulator and effector gene expression change in the same direction; uncoupling, in which regulator gene expression remains constant while effector gene expression changes; or inverse coupling, in which regulator and effector gene expression change in opposite directions. We have investigated the functional consequences of each form of coupling using a mathematical model to compare alternative circuits on the basis of engineering-inspired criteria for functional effectiveness. The results depend on whether the regulator protein acts as a repressor or activator of transcription at the promoters of effector genes. In the case of repressor control of effector gene expression, direct coupling is optimal among the three forms of coupling, whereas in the case of activator control, inverse coupling is optimal. Results also depend on the sensitivity of effector gene expression to changes in the level of a signal molecule; the optimal form of coupling can be physically realized only for circuits with sufficiently small sensitivity. These theoretical results provide a rationale for autoregulation of regulator genes in repressible gene circuits and lead to testable predictions, which we have compared with data available in the literature and electronic databases.

Keywords: gene regulation; repression; design principles; mathematical model; autoregulation

Introduction

Changes in bacterial gene expression in response to a signal are often mediated by the product of a regulator gene that coordinately regulates the expression of a set of effector genes. The regulator gene encodes a signal-responsive protein, such as the Trp repressor in *Escherichia coli*[1], that acts as an activator or repressor of transcription of the effector genes, and the effector genes encode enzymes and/or other types of effector molecules (e.g., flagellar proteins). The effector genes in many well-studied systems encode metabolic enzymes that are members of the same cellular pathway/system. When a signal molecule interacts with the regulator protein, the activity of the regulator protein is modified and effector gene expression changes.

The genes and gene products involved in the response to a signal are what make up a genetic regulatory circuit. Circuits can be classified into two types, repressible or inducible, based on the qualitative response to a signal. In a repressible circuit, an increase in the level of a signal molecule leads to a decrease in effector gene expression. For example, an increase in the cellular availability of tryptophan, which interacts with the Trp repressor, causes a decrease in expression of the Trp-regulated *trp* operon in *E. coli*, which encodes genes required for tryptophan biosynthesis[2]. The opposite occurs in an inducible circuit, the classical example of which is the lactose (*lac*) system in *E. coli*[2b]. The signal molecule in many well-studied systems is a metabolite that is a substrate and/or product of enzymes encoded by the co-regulated effector genes.

Circuits can also be classified based on the pattern of coupled regulator and effector gene expression. The regulator protein, independent of its function at the promoters of effector genes, can influence transcription of its own gene, a phenomenon that is called auotregulation[3; 4]. Thus, in

response to a signal, three patterns of coupled changes in regulator and effector gene expression are possible[5]: direct coupling, uncoupling, and inverse coupling. In a directly coupled circuit, changes in regulator and effector gene expression are coordinate. In an uncoupled circuit, regulator gene expression remains constant while effector gene expression changes. In an inversely coupled circuit, changes in regulator and effector gene expression are opposite. An example of a directly coupled circuit is the tryptophan (*trp*) system in *E. coli*[6], in which the level of regulator protein is repressible, just as the level of enzymes is repressible. An example of an uncoupled circuit is the asparagine (*asn*) system in *E. coli*[7; 8]. Unfortunately, an example of an inversely coupled circuit seems to be unavailable among repressible systems. However, inverse coupling has been documented among inducible systems[5], so we expect that this form of coupling is also relevant for repressible systems, at least as a formal possibility.

Faced with this diversity of circuit design, the following question arises[9; 10]. How do we explain the evolution of directly coupled, uncoupled, and inversely coupled circuits for repression? It is not immediately obvious why the level of regulator protein is repressible in some systems, constitutive in others, and possibly inducible in yet others, especially because the different types of circuits tend to have similar physiological functions. For example, the *trp* and *asn* circuits each spare the biosynthesis of an amino acid when the amino acid is available in the environment[2; 11]. Here, we address this question by comparing the functional capabilities of directly coupled, uncoupled, and inversely coupled circuits for control of repressible gene expression.

The present work extends the results of previous comparisons of direct coupling, uncoupling and inverse coupling in inducible gene circuits[5]. Although the possible forms of coupling in repressible

and inducible circuits are the same, the two types of circuits differ significantly. For example, repressible circuits tend to involve effector genes that encode biosynthetic enzymes, whereas inducible circuits tend to involve effector genes that encode catabolic enzymes; and of course, repressible and inducible circuits respond oppositely to signals. In a repressible circuit, effector gene expression is downregulated in response to an increase in the level of a signal molecule, whereas in an inducible circuit, effector gene expression is upregulated.

The present work also extends earlier more narrow comparisons[12; 13; 14; 15; 16] of repressible circuits. In this earlier work, two kinds of repressible circuits with special forms of coupling were considered: completely uncoupled circuits, in which the regulator protein has no effect whatsoever on regulator gene expression, and perfectly coupled circuits, in which relative changes in the expression of regulator and effector genes are identical because, for example, regulator and effector genes reside within the same operon. The more general forms of coupling considered here are more common than the completely uncoupled and perfectly coupled patterns of regulator and effector gene expression.

The foundation of the approach that we take to compare circuits is the method of controlled mathematical comparison[17; 18]. This method is implemented, in part, by defining *a priori* criteria for functional effectiveness[13] and by developing a generalized mathematical model for the different types of systems under consideration. Special cases of the model represent repressible gene circuits with each form of coupling. Systems that are compared are allowed to differ in ways that distinguish alternative circuit designs but are otherwise required to be as much alike as possible. The criteria for functional effectiveness, which are inspired partly by the properties of well-

designed man-made controllers, are used to score the results of comparisons. If differences in functional effectiveness are observed between different types of systems, we can hypothesize that these differences explain the evolution of the different circuit designs. On the other hand, if no differences are observed, we can hypothesize that diversity in circuit design is explained by historical accident, which is certainly important in some cases[19; 20]. The method of controlled mathematical comparison is a precise way to discover design principles of biological regulatory networks within the context of evolution and the limitations of tinkering[9].

The results of our comparisons indicate that there are functional differences associated with the different forms of coupling. For example, for systems in which the regulator protein is a repressor of effector gene expression (i.e., for repressor-controlled systems), we find that direct coupling allows faster responsiveness to signals than uncoupling, which in turn allows faster responsiveness than inverse coupling. These results lead to testable predictions. The predictions, which are similar to those for inducible systems[5], can be tested to a limited extent by comparing them with data available in the literature and electronic databases. If our predictions are supported by further tests, then we will have identified design principles of repressible gene circuits.

Theory

In this study we wish to understand what gives rise to differences in control of regulator gene expression in repressible gene circuits. Our study is carried out as follows. (1) We develop a generalized mathematical model for repressible gene circuits; a system with any of the three forms of coupling can be described by a special case of this model. (2) We next select systems for comparison. These systems differ in transcriptional control but are otherwise the same. Separate

comparisons are made for systems with activator and repressor control of effector gene expression, because this feature of a gene circuit (i.e., the mode of effector gene regulation) appears to be selected on the basis of environmental demand for effector gene expression[21]. (3) In comparisons, we determine the relative functional capabilities of alternative systems using well-defined *a priori* criteria for functional effectiveness[13]. We also determine how physical constraints on kinetic orders [22] influence the results.

Model

Equations and Variables

We will consider systems characterized by Fig. 1. A regulator gene encodes a regulator protein, which is a transcription factor, and the effector genes regulated by this protein encode enzymes that participate in biosynthesis of a metabolic end-product. The effector genes are coordinately regulated such that relative changes in gene expression are the same for each effector gene. The regulator protein can act as either a repressor or activator; its influence on transcription can be antagonized, unaffected (at the promoter of the regulator gene but not at the promoter of an effector gene), or stimulated by end-product binding; and its mode of regulation and sensitivity to end-product binding can depend on whether the regulator protein acts at the promoter of a regulator or effector gene. Because we are interested in coupling of regulator and effector gene expression in repressible circuits, we will only consider transcriptional circuitry for which an increase (decrease) in intracellular end-product concentration causes a decrease (increase) in effector gene expression As illustrated in Fig. 1, processes that we will consider include 1) synthesis of effector mRNA, 2) degradation of effector mRNA, 3) synthesis of effector enzymes, 4) dilution of effector enzymes through cell growth, 5) synthesis of end-product, 6) consumption of end-product, 7) cellular import

of end-product, 8) synthesis of regulator mRNA, 9) degradation of regulator mRNA, 10) synthesis of regulator protein, and 11) dilution of regulator protein. The metabolic pathway leading to end-product is taken to be unbranched, controlled by feedback inhibition, and composed of fast intermediate steps such that we can treat the pathway as a one-step reaction from initial substrate to final end-product. Note that the model considered here, which has been tailored to incorporate features of repressible circuits (e.g., end-product feedback inhibition), differs from the model used to compare direct coupling, uncoupling, and inverse coupling in inducible circuits[5].

The state of a system is characterized by the five dependent variables $X_1 ... X_5$, which represent the intracellular concentrations of effector mRNA (X_1), enzyme (X_2), end-product (X_3), regulator mRNA (X_4), and regulator protein (X_5). The independent variables $X_6 ... X_9$ are considered to be set by factors external to the system. These independent variables represent the precursor pools for mRNA and protein synthesis (X_6 and X_7), the amount of substrate available for conversion to end-product (X_8), and the amount of end-product available in the extracellular environment (X_9). We treat X_6 and X_7 as constants, and we will consider the effects of step changes in the availability of substrate (X_8) and exogenous end-product (X_9).

Based on Fig. 1, we derive a system of equations that includes one equation for each dependent variable, with each equation having the form $dX_i/dt = V_{+i} - V_{-i}$. Here, dX_i/dt is the rate at which concentration X_i changes with time t, V_{+i} is the net mass-flux directed into the pool characterized by X_i, and V_{-i} is the net mass-flux directed out of the pool characterized by X_i. We use the power-law formalism[23; 24] to specify rate laws for each flux V_{+i} and V_{-i}: Thus, we obtain

$$
\begin{aligned}
dX_1/dt &= \alpha_1 X_6^{g_{16}} X_3^{g_{13}} X_5^{g_{15}} - \beta_1 X_1^{h_{11}} \\
dX_2/dt &= \alpha_2 X_7^{g_{27}} X_1^{g_{21}} - \beta_2 X_2^{h_{22}} \\
dX_3/dt &= \alpha_3 X_8^{g_{38}} X_9^{g_{39}} X_2^{g_{32}} X_3^{g_{33}} - \beta_3 X_3^{h_{33}} \\
dX_4/dt &= \alpha_4 X_6^{g_{46}} X_3^{g_{43}} X_5^{g_{45}} - \beta_4 X_4^{h_{44}} \\
dX_5/dt &= \alpha_5 X_7^{g_{57}} X_4^{g_{54}} - \beta_5 X_5^{h_{55}}
\end{aligned}
\tag{1}
$$

in which the α and β parameters are rate constants that have strictly positive values, and the *g* and *h* parameters are real-valued kinetic orders: a negative value indicates an inhibitory influence, a positive value indicates a stimulatory influence, and a zero value indicates the absence of influence. The kinetic order g_{ij} describes the influence of concentration X_j on the flux V_{+i}, whereas the kinetic order h_{ij} describes the influence of concentration X_j on the flux V_{-i}.

One of the steady states of Eq. (1) is the steady state at the threshold of repression, i.e., the steady-state operating point at which effector gene expression is maximal. Note that we consider Eq. (1) to apply only over a certain range of end-product concentration and that we consider effector gene expression to be maximal at the low end of the regulatable range of end-product concentration (see supplementary material). We denote the steady-state concentrations at the threshold of repression as $(X_{10},\ldots,X_{90})$ and the steady-state fluxes as $(V_1,\ldots,V_5)$. Note that forward and reverse fluxes are equal at steady state. It is convenient to define the following dimensionless concentrations, which are normalized at the threshold of repression: $u_i = X_i/X_{i0}$ for $i = 1,\ldots,9$. Likewise, it is convenient to define the following turnover numbers: $F_i = V_i/X_{i0}$ for $i = 1,\ldots,5$. See the supplementary material for further discussion.

Parameters

Repressible circuits. We consider a circuit to be repressible if the steady-state level of effector protein decreases (increases) when the intracellular level of end-product increases (decreases). Thus, repressible circuits correspond to systems marked by a negative value of the steady-state logarithmic gain L_{23}[†], which is defined as $\partial \log X_2 / \partial \log X_3$:

$$L_{23} = \frac{g_{21}}{h_{11} h_{22}} \left(g_{13} + \frac{g_{15} g_{43}}{h_{44} h_{55} / g_{54} - g_{45}} \right) \tag{2}$$

The gain L_{23} characterizes the sensitivity of effector protein levels to changes in the intracellular supply of end-product. The expression for L_{23} in Eq. (2) is obtained from the steady-state solution of Eq. (1). Further discussion of L_{23} is provided in the supplementary material.

Activator and repressor control. The parameters describing the effect of a change in the concentration of the regulator protein on effector and regulator gene expression are g_{15} and g_{45}, respectively (Fig. 1). If g_{15} is positive, then the regulator protein is an activator of effector expression, and the system is considered to be positively regulated, i.e., activator controlled. If, on the other hand, g_{15} is negative, then the regulator protein is a repressor of effector expression, and the system is considered to be negatively regulated, i.e., repressor controlled. The kinetic order g_{45} similarly determines whether the regulator protein is an activator or repressor of regulator gene expression: systems with positive g_{45} are positively autoregulated, and systems with negative g_{45} are negatively autoregulated.

Form of coupling. The logarithmic gain $L_{53} = \partial \log X_5 / \partial \log X_3$ characterizes the sensitivity of the level of regulator protein to changes in the concentration of end-product:

$$L_{53} = g_{43} / \left(h_{44} h_{55} / g_{54} - g_{45} \right) \tag{3}$$

Like L_{23}, this gain is derived from the steady-state form of Eq. (1). Note that the signs of L_{53} and g_{43} are the same if $g_{45} < h_{44}h_{55}/g_{54}$, which, as we will see, is necessarily true for systems with a stable steady state. A system's form of coupling is determined by comparing the signs of L_{53} and L_{23}: if they have the same sign (i.e. $L_{53} < 0$), the system is directly coupled; if they have opposite sign (i.e. $L_{53} > 0$), the system is inversely coupled; and if $L_{53} = 0$, the system is uncoupled. Recall that $L_{23} < 0$ for systems of interest. See the supplementary material for further discussion of the gain L_{53}.

Physical limitations on kinetic orders that characterize regulation of gene expression. We will compare systems that differ in transcriptional control, focusing on differences in the kinetic orders g_{13} and g_{15}, which characterize regulation of effector gene expression, and the kinetics orders g_{43} and g_{45}, which characterize regulation of regulator gene expression (Fig. 1). The magnitudes of these kinetic orders are limited for physical reasons [22]. Thus,

$$\begin{aligned} g_{13} &\le \left| g_{13} \right|_{\max} \\ g_{15} &\le \left| g_{15} \right|_{\max} \\ g_{43} &\le \left| g_{43} \right|_{\max} \\ g_{45} &\le \left| g_{45} \right|_{\max} \end{aligned} \tag{4}$$

where $|g_{13}|_{max}$, $|g_{15}|_{max}$, $|g_{43}|_{max}$, and $|g_{45}|_{max}$ are small positive integers. We will consider kinetic orders to have magnitudes no greater than two or four.

We compare the different circuit designs (directly coupled, uncoupled, and inversely coupled) in a specific background, i.e., in the context of particular values for the non-regulatory parameters. Our estimates of these non-regulatory parameters are given in Table 1.

Equivalence conditions for controlled comparisons

We compare systems with the different forms of coupling under conditions of internal and external equivalence[17; 18]. This procedure is meant to ensure that any functional difference between alternative systems can be attributed to their differences in control of gene expression.

For internal equivalence, we require alternative systems to be identical except in the process of mRNA synthesis. Thus, only α_1, g_{13}, g_{15}, g_{16}, α_4, g_{43}, g_{45}, and g_{46}, which characterize transcription and transcriptional control, can have values that differ for two systems.

For external equivalence, we require that alternative systems exhibit the same steady-state response of enzyme level to changes in the level of intracellular end-product. This requirement is met if alternative systems have the same derepressed steady-state concentrations $X_{10}…X_{50}$ and have the same steady-state logarithmic gain L_{23}. We will consider activator- and repressor-controlled systems separately because of demand theory[21]. As a result, we also require alternative systems to have the same sign for the kinetic order g_{15}.

The first step in a comparison is to choose a reference system, the parameter values of which we will denote using primes. This reference system has a particular derepressed steady state (X'_{10},…, X'_{50}), gain L'_{23}, and sign of g'_{15}. These quantities and the reference system's non-regulatory parameter values (Table 1) are then used to determine the allowable parameter values for alternative systems. Internal equivalence fixes the values of all but eight parameters, which are α_1, g_{13}, g_{15}, g_{16}, α_4, g_{43}, g_{45}, and g_{46}. However, because X_6 is a constant, the terms $\alpha_1 X_6^{g16}$ and $\alpha_4 X_6^{g46}$ in Eq. (1) can each be treated as a single lumped parameter, which effectively leaves only six free parameters. External equivalence imposes further constraints. Equivalent systems are required to have the same steady state at the threshold of derepression, from which it follows that $V_1 = V'_1$ and $V_4 = V'_4$ for any system equivalent to the reference system. These two constraints, Eq. (1), and the parameter values of the reference system determine the values of $\alpha_1 X_6^{g16}$ and $\alpha_4 X_6^{g46}$ for equivalent systems. Furthermore, Eq. (2) and the requirement that $L_{23} = L'_{23}$ fixes one of the four kinetic orders that characterize transcriptional control once the other three kinetic orders are specified. Thus, to examine a family of equivalent systems, it is sufficient to vary only three parameters (e.g., g_{15}, g_{43}, and g_{45}). See the supplementary material for further discussion.

Functional effectiveness

We compare equivalent systems using five *a priori* criteria for functional effectiveness[13]: selectivity, stability, robustness, efficiency, and temporal responsiveness. Each criterion is described below.

A system is considered to be selective if $|L_{53}|$ is less than a threshold value[5; 22], such that the steady-state level of regulator protein is sufficiently insensitive to changes in the level of end-product. Limited variation of regulator gene expression avoids three potential problems: nonspecific interactions with unrelated systems when the level of regulator protein is high, loss of regulation when the level of regulator protein is low, and possible variation in cellular growth rate caused by large changes in the burden of protein synthesis.

A system is considered to be effective with respect to stability if its derepressed steady state is locally stable and characterized by a large margin of stability[13]. Local stability ensures that the system returns to its steady state following a small disturbance of this state, and a large margin of stability ensures that the system's steady state remains stable despite small changes in the system's structure (i.e., the system's parameter values).

To determine if the derepressed steady state of Eq. (1) is locally stable, we study the associated linearization[25; 26]: $d\mathbf{x}/dt = \mathbf{L}\mathbf{x}$, where $\mathbf{x} = (x_1,\ldots,x_5)^T$ and $x_i = u_i - 1$. The elements of $\mathbf{L}$ are defined as $l_{ij} = -F_i(h_{ij} - g_{ij})$. If the characteristic polynomial of $\mathbf{L}$, $p(\lambda) \equiv \det(\mathbf{L} - \lambda\mathbf{I})$, has roots all with negative real parts (i.e., if $p(\lambda)$ is Hurwitz), then the steady state of Eq. (1) is locally stable[25; 27]. The characteristic polynomial of $\mathbf{L}$ is

$$\begin{aligned} p(\lambda) &= \lambda^5 + a_1\lambda^4 + a_2\lambda^3 + a_3\lambda^2 + a_4\lambda + a_5 \\ &= \left[(\lambda + f_1)(\lambda + f_2)(\lambda + f_3) - c_1 g_{13}\right]\left[(\lambda + f_4)(\lambda + f_5) - c_2 g_{45}\right] - c_1 c_2 g_{15} g_{43} \end{aligned} \tag{5}$$

where $c_1 = F_1F_2F_3g_{21}g_{32}$, $c_2 = F_4F_5g_{54}$, $f_1 = F_1h_{11}$, $f_2 = F_2h_{22}$, $f_3 = F_3(h_{33} - g_{33})$, $f_4 = F_4h_{44}$, and $f_5 = F_5h_{55}$. See the supplementary material for further discussion.

To determine if $p(\lambda)$ is Hurwitz, we use the Liénard-Chipart criterion[25; 27]. This criterion states that $p(\lambda)$ is Hurwitz if and only if $a_1 > 0$, $a_3 > 0$, $a_5 > 0$, $\Delta_2 > 0$, and $\Delta_4 > 0$, where a_1, a_3, and a_5 are coefficients of $p(\lambda)$ (Eq. (5)) and Δ_2 and Δ_4 are Hurwitz determinants[25; 28]: $\Delta_2 = a_1a_2 - a_3$ and $\Delta_4 = (a_1a_2 - a_3)(a_3a_4 - a_5) - (a_1a_4 - a_5)^2$. Note that $a_5 > 0$ implies $g_{45} < h_{44}h_{55}/g_{54}$[‡]. We will focus on systems that satisfy this necessary condition for stability, which simplifies the analysis of results.

A system's margin of stability is defined as a distance in parameter space, the distance between the point representing the system and the closest point representing a system with an unstable steady state. The parameter space of interest is the space of g_{13} and g_{45}, because it can be shown that each parametric term in Eq. (5) is the same for equivalent systems with the exceptions of g_{13}, g_{45}, and the linkage coefficient[29] $g_{15}g_{43} = (L_{23}h_{11}h_{22}/g_{21} - g_{13})(h_{44}h_{55}/g_{54} - g_{45})$, which is a function of g_{13}, g_{45}, and quantities that are the same for equivalent systems. See the supplementary material for further discussion.

There are two curves in the (g_{13}, g_{45})-parameter space that are of importance for determining the margin of stability: the divergence curve, along which $a_5 = 0$, and the unstable oscillation curve, along which $\Delta_4 = 0$. Systems with stable steady states are bounded by these two curves (Fig. 2). The divergence curve, which is so called because points on this curve correspond to a zero eigenvalue, is the straight line $g_{45} = h_{44}h_{55}/g_{54}$. The unstable oscillation curve, which corresponds to points with a pair of conjugate pure imaginary eigenvalues, is an algebraic curve of order three, which follows from the expression given above for Δ_4 . Thus, the margin of stability can be calculated as the shortest distance between a (stable) system of interest and either of these curves.

We will use the Manhattan metric to measure distance. See the supplementary material for further discussion.

A system is considered to be robust if its steady states are characterized by parameter sensitivities with small magnitudes[13; 30]. Parameter sensitivities with small magnitudes indicate that a system's steady state is insensitive to disturbances that affect the system's structure (i.e., the system's parameter values)[31].

Sensitivities are defined as follows. The sensitivity of the steady-state concentration X_i to a change in a parameter p is defined as $S(X_i,p) = \partial \log X_i / \partial \log p$, where p can be a rate constant or kinetic order. Likewise, the sensitivity of the steady-state flux V_i to a change in a parameter p is defined as $S(V_i,p) = \partial \log V_i / \partial \log p$. Expressions for X_i and V_i are found from the steady-state form of Eq. (1), which can be written as $\mathbf{Ay} = \mathbf{b} + \mathbf{i}$. Here, $\mathbf{i}$ is a vector involving the independent concentrations (X_6, X_7, X_8, and X_9), $\mathbf{b}$ is a vector defined as $(b_1,\ldots,b_5)^T$ in which each $b_i = \log \beta_i / \log \alpha_i$, $\mathbf{y}$ is a vector defined as $(y_1,\ldots,y_5)^T$ in which each $y_i = \log X_i$, and $\mathbf{A}$ is a matrix with elements $a_{ij} = g_{ij} - h_{ij}$. All parameter sensitivities are proportional to sensitivities of the form $S(X_i,\beta_j)$[32], which compose the elements of $\mathbf{A}^{-1}$. See the supplementary material for further discussion.

As explained elsewhere[13], a system is considered to be efficient if it produces appropriate steady-state input-output behavior. We are concerned with the steady-state responses of effector protein (X_2) and intracellular end-product (X_3) to three types of changes: (1) changes in the level of substrate (X_8); (2) changes in the level of exogenous end-product (X_9); and (3) changes in the rate of end-product consumption (β_3). The relevant quantities that characterize responses to these

changes are the following gains and sensitivities: L_{28}, L_{38}, L_{29}, L_{39}, $S(X_2, \beta_3)$ and $S(X_3, \beta_3)$. Each of these gains and sensitivities can be related to the gain L_{23} as follows:

$$\begin{aligned} \frac{L_{28}}{g_{38}} &= \frac{L_{29}}{g_{39}} = -S(X_2, \beta_3) = \frac{L_{23}}{h_{33} - g_{33} - g_{32} L_{23}} \\ \frac{L_{38}}{g_{38}} &= \frac{L_{39}}{g_{39}} = -S(X_3, \beta_3) = \frac{1}{h_{33} - g_{33} - g_{32} L_{23}} \end{aligned} \tag{6}$$

A system is considered to be temporally responsive if it has a small settling time following a step decrease in the level of substrate (X_8) or exogenous end-product (X_9). Settling time is defined as the time required for the level of enzyme to approach and remain within 5% of its steady-state value. Time-courses of derepression are found by using standard methods[33] to numerically solve the following initial value problem, which is derived from Eq. (1):

$$\begin{aligned} \varepsilon\, du_1/d\tau &= u_3^{g_{13}} u_5^{g_{15}} - u_1 \\ du_2/d\tau &= u_1 - u_2 \\ \mu\varepsilon\, du_3/d\tau &= f_r u_2^{g_{32}} u_3^{g_{33}} - u_3^{h_{33}} \\ \varepsilon\, du_4/d\tau &= u_3^{g_{43}} u_5^{g_{45}} - u_4 \\ du_5/d\tau &= u_4 - u_5 \end{aligned} \tag{7}$$

where

$$f_r(\tau) = u_8^{g_{38}} u_9^{g_{39}} = \begin{cases} f_{r,\max} & \text{if } \tau < 0 \\ f_{r,\min} & \text{if } \tau \geq 0 \end{cases} \tag{8}$$

The initial condition is given by

$$\begin{aligned} u_1(0) = u_2(0) &= \left(f_{r,\max}\right)^{E_2/E_3} \\ u_3(0) &= \left(f_{r,\max}\right)^{1/E_3} \\ u_4(0) = u_5(0) &= \left(f_{r,\max}\right)^{E_1/E_3} \end{aligned} \tag{9}$$

Recall that the variables u_i are normalized concentrations X_i/X_{i0} and that the parameters F_i are turnover numbers V_i/X_{i0}. Several new quantities are introduced in Eqs. (7)-(9). Eq. (7) is obtained from Eq. (1) by setting $X_6 = X_{60}$, $X_7 = X_{70}$, $g_{21} = 1$, $g_{54} = 1$, $h_{11} = 1$, $h_{22} = 1$, $h_{44} = 1$, $h_{55} = 1$, $F_4 = F_1$, and $F_5 = F_2$ (Table 1) and by introducing the dimensionless variables $\varepsilon = F_2/F_1$, $\mu = F_1/F_3$, and $\tau = F_2 t$. The dimensionless repression factor f_r, which is related to X_8 and X_9, is defined in Eq. (8). The parameters in the exponents of Eq. (9) are defined as follows: $E_1 = g_{43}/(1 - g_{45})$, $E_2 = g_{13} + g_{15}E_1$, and $E_3 = h_{33} - g_{33} - g_{32}E_2$. As indicated in Eq. (8), we follow the dynamics of deprepression initiated by a step decrease in the repression factor f_r from $f_{r,\max}$ to $f_{r,\min}$. The final derepressed steady state corresponds to $f_r = f_{r,\min}$, which we set equal to 1 without loss of generality. As indicated in Eq. (9), the initial repressed steady state is determined by the value of $f_{r,\max}$, which is 100 for all cases considered (Table 1).

We compare the responsiveness of systems with a particular gain $L_{23} < 0$ and a particular sign for $g_{15} \neq 0$ by considering families of systems in the (g_{15}, g_{43}, g_{45})-parameter space. For each of several planes of consant g_{15} in this space, we find three settling times: the settling time of uncoupled circuits ($t^{(U)}$, $g_{43} = 0$), the minimal settling time for directly coupled circuits ($t_{\min}^{(D)}$, $g_{43} < 0$), and the minimal settling time for inversely coupled circuits ($t_{\min}^{(I)}$, $g_{43} > 0$). To find the minimal settling times $t_{\min}^{(D)}$ and $t_{\min}^{(I)}$, we sample the regions of parameter space where directly and inversely coupled circuits are represented by (pseudo) randomly selecting values for g_{43} and g_{45} within the ranges allowed by Eq. (4). Note that uncoupled systems have settling times that are independent of g_{45}. Results are obtained for a variety of values of L_{23} and g_{15}[§].

To determine how comparisons depend on estimates of turnover numbers, for each family of systems above, we obtained and compared the settling times $t^{(U)}$, $t_{min}^{(D)}$, and $t_{min}^{(I)}$ for systems with (pseudo) randomly generated values for the turnover numbers F_1 ($= F_4$), F_2 ($= F_5$), and F_3. Sampling of each turnover number was centered on the estimated value given in Table 1 and log uniform in the range of 10% to 1000% of the estimated value.

Results

Below, we compare directly coupled, uncoupled, and inversely coupled circuits that are described by the model illustrated in Fig. 1. We begin by considering the constraints that limit the magnitudes of kinetic orders (Eq. (4)). We then report detailed results for comparisons of circuits based on selectivity, stability, robustness, efficiency, and temporal reponsiveness. The comparisons depend in part on an assumption of specific parameter estimates (Table 1).

Physical limitations on the form of coupling

We find that a constraint on the magnitude of the kinetic order g_{13} (Eq. (4)) can limit the form of coupling in a system. The limitation follows from Eqs. (2)-(4) and our requirements that $g_{15} \neq 0$ and $L_{23} < 0$, and it depends on the relationship between L_{23} and a critical gain L_{23}^*, which is defined as $-|g_{13}|_{max}/(h_{11}h_{22}/g_{21})$:

$$\begin{aligned} L_{23} < L_{23}^* &\Leftrightarrow g_{15}L_{53} < -g_{13} - |g_{13}|_{\max} \\ L_{23} = L_{23}^* &\Leftrightarrow g_{15}L_{53} = -g_{13} - |g_{13}|_{\max} \\ L_{23} > L_{23}^* &\Leftrightarrow g_{15}L_{53} > -g_{13} - |g_{13}|_{\max} \end{aligned} \qquad (10)$$

Equation (10) simply indicates that $g_{15}L_{53}$ is negative when $|L_{23}| > |L_{23}^*|$, negative or zero when $|L_{23}| = |L_{23}^*|$, and negative, zero, or positive when $|L_{23}| < |L_{23}^*|$. Thus, for systems with high gain (i.e., $|L_{23}| > |L_{23}^*|$), only inverse coupling ($L_{53} > 0$) is possible with repressor control of effector gene expression ($g_{15} < 0$), and only direct coupling ($L_{53} < 0$) is possible with activator control of effector gene expression ($g_{15} > 0$). For systems with intermediate gain (i.e., $|L_{23}| = |L_{23}^*|$), uncoupling ($L_{53} = 0$) is also possible, and for systems with low gain (i.e., $|L_{23}| < |L_{23}^*|$), each form of coupling is possible. The above results are closely related to those obtained in an earlier study for inducible circuits[5]. For further discussion, see the supplementary material.

Selectivity

Systems that have $|L_{53}|$ below a certain threshold are selective. To estimate an upper bound on $|L_{53}|$, we make a number of assumptions. We assume that more than one molecule per cell is required to maintain regulation, and fewer than 1000 molecules of regulator protein per cell are required to avoid dysfunctional crosstalk or an excessive protein burden on the cell. We also assume that regulation takes place over an approximate 30-fold range of end-product concentration. From these assumptions, we find that the upper bound on the magnitude of L_{53} is two, which implies that a directly coupled or inversely coupled system has the potential to be selective if $|L_{53}| \leq 2$ but not otherwise. If $|L_{53}| > 2$, the level of regulator protein can fall below one molecule per cell, which abolishes selectivity, or rise above 1000 molecules per cell, which also abolishes selectivity. Thus, if $|L_{53}| \leq 2$, each form of coupling allows for selectivity. Note that systems with strong negative autoregulation of the regulator gene will tend to be selective, because decreasing g_{45} decreases the magnitude of L_{53} (Eq. (3)).

Stability

We find that negative autoregulation of the regulator gene favors stability and that the form of coupling has little impact on stability, especially when negative autoregulation of the regulator gene is strong. In Fig. 2, equivalent systems with the parameter values of Table 1 can be compared graphically on the basis of stability. Systems with a stable steady state are represented in a region of (g_{13}, g_{45})-parameter space bounded by the divergence ($a_5 = 0$) and unstable oscillation ($\Delta_4 = 0$) curves; systems with an unstable steady state are represented in the shaded region. As can be seen, most systems lie closer to the divergence curve than the unstable oscillation curve. We use the Manhattan metric to measure distance, but the same qualitative result is obtained using other metrics. Among the systems closer to the divergence curve, which include systems with each form of coupling, systems with identical g_{45} have the same margin of stability, because they are equidistant from the divergence curve, the nearest boundary of instability. It can be further seen that the margin of stability increases as g_{45} decreases, which also holds for systems that are closer to the unstable oscillation curve than the divergence curve. Thus, it seems that the three forms of coupling each allow the same margin of stability, and any difference in margin of stability between two systems can be reduced by decreasing g_{45}. We conclude that the different forms of coupling are indistinguishable on the basis of our stability criterion and that stability is promoted by strong negative autoregulation (i.e. negative values of g_{45} of large magnitude), at least for our estimates of non-regulatory parameters.

Robustness

Our analysis shows that robustness is enhanced by negative autoregulation of regulator gene expression and that the different forms of coupling allow similar robustness. The robustness of a system's steady state is measured by parameter sensitivities, which were calculated numerically using the parameter estimates of Table 1. Results for sensitivities of the form $S(X_i, \beta_j)$ are shown in Fig. 3 for repressor-controlled systems. Recall that other sensitivities are proportional to these sensitivities[32]. As illustrated in Fig. 3(a), equivalent systems with direct coupling, uncoupling, and inverse coupling can be found that have similar sensitivities. Moreover, sensitivities involving X_2 (enzyme) and X_3 (end-product), which are likely to have greater physiological importance than sensitivities involving X_1, X_4, or X_5, are the same for each system. As can be seen by comparing panels (b) and (c) of Fig. 3, differences between equivalent systems with the different forms of coupling are reduced overall when the value of g_{45} shared by these systems is decreased, which indicates that negative autoregulation promotes robustness. Note that the systems considered in Fig. 3 have robust steady states: a 1% change in any of the rate constants β_j leads to less than a 1% change in any of the concentrations X_i. Similar results are obtained for activator-controlled systems (not shown). A more detailed analytical comparison supports the numerical results and confirms that negative autoregulation is associated with increased robustness (see supplementary material). It seems that there is no necessary distinction among the different forms of coupling on the basis of our robustness criterion.

Efficiency

The efficiency of a system is determined by the logarithmic gains and parameter sensitivities in Eq. (6). Because equivalent systems have the same non-regulatory parameter values and the same gain L_{23}, the gains and sensitivities of Eq. (6) are identical for equivalent systems, and directly

coupled, uncoupled and inversely coupled systems are equal with respect to our efficiency criterion.

Temporal responsiveness

We find significant differences in temporal responsiveness for systems with different types of coupling. Temporal responsiveness requires that the level of effector enzyme adjust quickly to changes in the level of substrate or exogenous end-product. Time courses triggered by such a change are shown in Fig. 4 for systems with repressor (panel (a)) and activator (panel (b)) control of effector gene expression. Settling times calculated for larger sets of repressor- and activator-controlled systems are represented in Fig. 5.

Let us first consider repressor-controlled systems. In Fig. 4(a), we see that settling time increases monotonically as the gain L_{53} increases: $\tau_1 < \tau_2 < \tau_3 < \tau_4 < \tau_5$, where τ_i denotes the dimensionless settling time $\tau_i = F_2 t_i$ associated with time course (i). Time courses (1) and (2) correspond to directly coupled systems with $L_{53} = -1/3$ and $-1/6$, respectively; time course (3) corresponds to an uncoupled system with $L_{53} = 0$; and time courses (4) and (5) correspond to inversely coupled systems with $L_{53} = 1/6$ and $1/3$, respectively. These results suggest that, for a repressor controlled system, direct coupling ($L_{53} < 0$) allows faster responsiveness than uncoupling ($L_{53} = 0$), which in turn allows faster responsiveness than inverse coupling ($L_{53} > 0$). This ordering of the three forms of coupling with respective to responsiveness is supported by exhaustive calculations of settling times for equivalent systems within each of various families of equivalent systems. Representative results are shown in Fig. 5(a). This contour plot shows settling times for systems represented within a cross-section of the parameter space (g_{15}, g_{43}, g_{45}) in which $g_{15} < 0$ is constant. Settling

times are shown only for stable systems that satisfy the constraints of Eq. (4). It is apparent that systems with large negative g_{45} (i.e. strong negative autoregulation) have faster settling times. Also, as expected from the trend seen in Fig. 4(a), the minimum settling time within this cross-section is located where $g_{43} < 0$, which is indicative of direct coupling (Eq. (3)). It can also be seen that systems with $g_{43} = 0$ (uncoupling) have a faster settling time than systems with $g_{43} > 0$ (inverse coupling). We investigated the sensitivity of these results to uncertainty in our estimates of turnover numbers. The results indicate that the ordering found on the basis of the estimates in Table 1 remains the same for an overwhelming majority of the parameter sets randomly selected for consideration (see supplementary material).

Let us now consider activator-controlled systems. The values of L_{53} that produce time courses (i) in Fig. 4(a) and ($i + 5$) in Fig. 4(b) are the same for $i = 1,\ldots,5$. Inspection of time courses (7) – (9), which correspond to systems with direct coupling (7), uncoupling (8), and inverse coupling (9), suggest that the trend of Fig. 4(a) is perhaps reversed. In other words, for these cases, settling time increases, rather than decreases, as the gain L_{53} decreases: τ_9 (= 0.53) < τ_8 (= 1.45) < τ_7 (= 1.48). However, time courses (6) and (10) are inconsistent with a simple reversal in trend. The settling time of time course (6), $\tau_6 = 1.19$, is smaller, not larger, than that of time course (7), and because of overshoot, the settling time of time course (10), $\tau_{10} = 2.18$, is larger, not smaller, than that of time course (9). Greater insight is obtained by exhaustive calculations of settling time for equivalent systems within each of various families of equivalent systems. Typical results are shown in Fig. 5(b). As for repressor-controlled systems, activator-controlled systems with large negative g_{45} (i.e. strong negative autoregulation) have faster settling times. It can be seen that the minimal settling time in this plot corresponds to a system with $g_{43} > 0$ (inverse coupling). It can also be seen that

some systems with $g_{43} < 0$ (direct coupling) have a settling time less than that of a system with $g_{43} = 0$ (uncoupling). These and other results (not shown) suggest the following conclusions. For an activator controlled system, inverse coupling allows faster responsiveness than direct coupling, which in turn allows faster responsiveness than uncoupling. The robustness of this ordering was investigated to determine the effect of uncertainty in estimates of turnover numbers. Representative results are shown in Fig. 6 and support the above ordering. As indicated in Fig. 6(a), inverse coupling is expected to allow faster responsiveness than direct coupling; as indicated in Fig. 6(b), inverse coupling is expected to allow faster responsiveness than uncoupling; and as indicated in Fig. 6(c), direct coupling is expected to allow faster responsiveness than uncoupling.

Discussion

A number of genes in *E. coli*, and other bacteria, are regulated in response to changes in the availability of small-molecule metabolites. Genes that are turned off in the presence of a metabolite are said to have a repressible pattern of expression, whereas genes that are turned on are said to have an inducible pattern of expression. Here, we considered regulator gene expression in systems where effector gene expression is repressible. Using the method of controlled mathematical comparison[17; 18], we have attempted to determine whether the regulator gene should be expected to have a repressible (direct coupling), inducible (inverse coupling), or constitutive (uncoupling) pattern of expression. We have also considered whether the regulator should positively or negatively regulate its own gene, or alternatively have no effect at all.

Our results indicate that the regulator protein should generally be expected to negatively regulate its own gene, i.e., to act as a repressor at the promoter of its own gene. Negative autoregulation is

associated with selectivity, stability, robustness, and temporal responsiveness in repressible gene circuits. Consistent with the importance of negative autoregulation suggested here and in an earlier study[5], many of the regulator proteins in *E. coli* are negatively autoregulated[34]. Also, consistent with the results reported here and in earlier reports, experimental studies of synthetic gene circuits have indicated that negative autoregulation is associated with stability[35], robustness[30], and temporal responsiveness[36].

The expected type of coupling depends on whether the effector gene is under activator or repressor control. The functional consequences of changing the type of coupling are seen in analyzing the temporal responsiveness of alternative circuit designs. For repressor-controlled systems, we obtained results that are qualitatively the same as those obtained earlier for inducible systems with repressor control[5]: direct coupling allows faster responsiveness than uncoupling, which in turn allows faster responsiveness than inverse coupling. In contrast, for activator-controlled systems, we obtained results that are similar but qualitatively different from those obtained earlier for inducible systems with activator control[5]: inverse coupling allows faster responsiveness than direct coupling, which in turn allows faster responsiveness than uncoupling. For both repressible and inducible systems, inverse coupling is optimal, but direct coupling is superior to uncoupling in a repressible system[5]. These results are robust to changes in turnover numbers (Fig. 6).

Because qualitative results are the same for repressor-controlled systems regardless of whether effector genes are inducible or repressible but not for activator-controlled systems, we carefully examined the results for repressible systems under activator control. Although direct coupling allows a faster settling time than uncoupling, we find that the ability to establish a steady-state level

of enzyme faster comes at some cost: (1) there is an initial delay in enzyme response at early times, which is less for an uncoupled system, and (2) there is transient expression of the activator protein above its steady-state level. Activator overexpression can continue for several doubling times, which could be a disadvantage in rapidly changing environments. Thus, the relative effectiveness of directly coupled and uncoupled activator-controlled systems with respect to dynamics of response is more ambiguous than indicated by comparisons of setting times alone.

As predicted for inducible systems[5; 22] and in an earlier more limited study of repressible systems[15], not all types of coupling are generally physically realizable because of limits on the magnitudes of kinetic orders (Eqs. (4) and (10)). With repressor control, systems that have high gain ($|L_{23}| > |L_{23}^*|$) are limited to inverse coupling, and systems that have intermediate gain ($|L_{23}| = |L_{23}^*|$) are limited to inverse coupling or uncoupling. With activator control, systems that have high gain are limited to direct coupling, and systems that have intermediate gain are limited to direct coupling or uncoupling. Only systems that have low gain ($|L_{23}| < |L_{23}^*|$) are unconstrained with respect to the form of coupling.

The results of our comparisons allow us to formulate predictions for gene circuits with repressible effector genes. In repressible systems, we generally expect the regulator protein to negatively regulate its own expression, ensuring stability, robustness, selectivity and temporal responsiveness. Within this constraint, the optimal form of coupling is the most responsive one among the forms of coupling that are physically realizable, which is determined by the magnitude of the gain L_{23}. For repressor-controlled systems, we expect direct coupling if the gain is low, uncoupling if the gain is intermediate, and inverse coupling if the gain is high. For activator-controlled systems, we expect

inverse coupling if the gain is low and direct coupling if the gain is intermediate or high. These predictions are similar to those for inducible systems[5]. The only difference is that for repressible systems we never expect uncoupling with activator control, whereas we do for inducible systems with intermediate gain.

How well do these predictions compare with the historical results of evolution? There are over 400 promoter regions for which regulatory interactions have been documented in RegulonDB[37]. To test our predictions, we must first identify those systems, more or less consistent with Fig. 1, for which the mode of regulator action has been determined at the promoters of effector and regulator genes (i.e., systems for which the signs of g_{15} and g_{45} are known) and for which the signal molecule has been identified. Ideally, we would also have available for each system a direct measurement of the gain L_{23}. This gain is usually unavailable, but as discussed elsewhere in the context of inducible systems[5], we can expect the gain in effector gene expression to correlate with a more readily available measurement, the ratio of maximal to minimal level of effector gene expression, which has been called the capacity of effector gene expression. Although RegulonDB and a derivative[38] contain information about whether a regulator is an activator or repressor of a promoter, they do not contain detailed information about the influence of signal molecules. Thus, to test predictions, we must rely mostly on a careful reading of the primary literature. Our reading of the literature has uncovered about 10 repressible systems in bacteria that can serve as test cases for our predictions.

Let us first consider the examples of repressible activator-controlled genes. Regulator-effector gene pairs in this class include *asnC-asnA* in *E. coli*[7; 8], *cysB-cysP* in *E. coli* and *Salmonella*[39; 40], *fadR-fabA* in *E. coli*[41; 42; 43], and *fruR-ppsA* in *E. coli* and *Salmonella*[44; 45; 46; 47; 48]. Two of the four

regulator genes in these systems are autoregulated: *asnC* and *cysB*, both of which are negatively autoregulated. In each case, expression of the regulator gene is apparently unresponsive to the signal for repression/derepression of the effector gene. A constitutively expressed activator gene is inconsistent with our predictions, but an inversely coupled system may appear to be uncoupled unless experiments are designed to detect subtle changes in regulator gene expression. As can be seen in Fig. 5(b), responsive activator-controlled systems with inverse coupling tend to have a value for g_{43} that is small in magnitude and a value for g_{45} that is large in magnitude. Thus, from Eq. (3), we expect only modest changes in regulator gene expression. For inducible systems with inverse coupling, we have estimated that regulator gene expression may vary over less than a 2-fold range[5]. This estimate applies for repressible systems as well. Of course, the criteria for functional effectiveness considered here may not be the most relevant for the particular systems under consideration[5], or the predictions may not apply, because the systems are inadequately represented by the generic model of Fig. 1 and/or the parameter estimates of Table 1. The latter explanation might be relevant in the case of *asnC* and *asnA*. Recent data suggest that expression of these genes is perhaps inversely coupled through a mechanism more complicated than that considered in Fig. 1. In this system, *asnA* encodes an asparagine synthetase, the expression of which is controlled through AsnC in response to the level of asparagine. Expression of *asnC*, which is negatively autoregulated, appears to be unaffected by asparagine but is repressed by the nitrogen assimilation control (Nac) protein when ammonium is limiting[49]. Because ammonia is the nitrogen donor in AsnA-catalyzed asparagine biosynthesis[11], upregulation of *asnA* could potentially lead to Nac-mediated downregulation of *asnC*. In any case, because few examples of activator-controlled repressible genes are known, further tests of our predictions, as more data become available, seem necessary to reach conclusions about the relevance of the predictions.

Let us now consider the examples of repressible repressor-controlled genes. Regulator-effector gene pairs in this class include *trpR-trpLEDCBA* in *E. coli*[6; 50], *alaS* (AlaS has both regulator and effector functions) in *E. coli*[51], *metJ-metBL* in *E. coli* and *Salmonella*[52], *fur-iucABCD* in *E. coli*[53; 54; 55], *purR-purB* in *E. coli*[56; 57], *argR-argF* in *E. coli*[58], the *pyr* operon (the regulator gene *pyrR* is the first gene in this operon) in *Bacillus subtilis* and *Enterococcus faecalis*[59; 60; 61], *tyrR-aroF* in *E. coli*[62; 63], and *nadR-nadB* in *Salmonella*[64]. Eight of these systems exhibit negative autoregulation: only *nadR* is constitutively expressed. With the exception of the last two cases, which are characterized by an uncoupled pattern of expression, the experimental evidence indicates direct coupling. In each case, the capacity for effector gene expression is 50-fold or less, which suggests a low gain L_{23} for each system. Thus, seven of the nine examples seem consistent with our prediction of direct coupling in the case of low gain.

The survey of regulator gene expression in repressible systems summarized above and a similar survey for inducible systems[5] are consistent empirically. In systems, inducible or repressible, controlled by a repressor of effector gene expression, expression of the regulator protein tends to follow that of effector gene products (i.e., there is a pattern of direct coupling), whereas in systems controlled by an activator of effector gene expression, expression of the regulator protein tends to remain more constant during changes in effector gene expression (i.e., there is a real or apparent pattern of uncoupling). These surveys, which reveal that activator- and repressor-controlled systems exhibit distinct preferential forms of coupling, and that negative autoregulation is common, suggest that there are indeed rules that govern the pattern of regulator and effector gene expression. They also suggest that the system properties considered here and in earlier studies, particularly

temporal responsiveness, can explain the rules, at least in part, particularly for repressor-controlled systems.

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Figure legends

Fig. 1. Model. The numbered arrows represent mass fluxes. Other arrows represent catalytic or regulatory influences. The kinetic orders influencing gene regulation are g_{13}, $g_{15,}$ $g_{43,}$ and g_{45}. The kinetic order g_{13} characterizes the influence of the end-product on effector gene expression, and the kinetic order $g_{15} \neq 0$ characterizes the influence of the regulator protein on effector gene expression. Systems with positive (negative) g_{15} are under activator (repressor) control. The kinetic order g_{43} characterizes the influence of the end-product on regulator gene expression, and the kinetic order g_{45} characterizes the influence of the regulator protein on regulator gene expression. Systems with positive (negative) g_{45} have positive (negative) autoregulation; systems with $g_{45} = 0$ have a constitutively expressed regulator protein with no influence at the promoter of its own gene. We define a repressible system as one for which the steady-state gain $L_{23} = \partial \log X_2 / \partial \log X_3$ (Eq. (2)) is negative. The steady-state gain $L_{53} = \partial \log X_5 / \partial \log X_3$ (Eq. (3)) may be positive (inverse coupling), zero (uncoupling), or negative (direct coupling).

Fig. 2. Representation of systems with stable and unstable steady states in the parameter space of g_{13} and g_{45}. The domain that represents systems with a stable steady state is bordered by two boundaries of instability: the line along which $a_5 = 0$ and the algebraic curve of third degree along which $\Delta_4 = 0$. Systems with an unstable steady state lie in the shaded region; systems represented elsewhere in this plot have a stable steady state.

Fig. 3. Numerical parameter sensitivities of equivalent systems with alternative forms of coupling. In each panel, solid bars correspond to a directly coupled system (D), striped bars correspond to an uncoupled system (U), and open bars correspond to an inversely coupled system (I). Sensitivities of X_1, X_2, and X_3 are not shown in panels (b) and (c), as in panel (a), because these sensitivities are the same in both cases for the three systems considered. The results of panel (a) are based on $L_{23} = -0.5$; $g_{15} = -1$; $g_{45} = -1$; (D) $g_{43} = -1$, $g_{13} = -1$; (U) $g_{43} = 0$, $g_{13} = -0.5$; and (I) $g_{43} = 1$, $g_{13} = 0$. The results of panel (b) are based on $L_{23} = -0.5$; $g_{15} = -1$; $g_{45} = -1$; (D) $g_{43} = -0.667$, $g_{13} = -0.833$; (U) $g_{43} = 0$, $g_{13} = -0.5$; and (I) $g_{43} = 0.667$, $g_{13} = -0.167$. The results of panel (c) are based on $L_{23} = -0.5$; $g_{15} = -1$; $g_{45} = -2$; (D) $g_{43} = -1$, $g_{13} = -0.833$; (U) $g_{43} = 0$, $g_{13} = -0.5$; and (I) $g_{43} = 1$, $g_{13} = -0.167$. The values given in Table 1 were used for non-regulatory parameters in all calculations.

Fig. 4. Time courses of derepression. The parameter values in Table 1, in addition to $L_{23} = -1$ and $g_{45} = -2$, hold for all curves in both panels. For panel (a), $g_{15} = -2$, and for panel (b), $g_{15} = 2$. Values of g_{43} and g_{13} are as follows for curves (1) – (10): (1) $g_{43} = -1$, $g_{13} = -1.67$; (2) $g_{43} = -0.5$, $g_{13} = -1.33$; (3) $g_{43} = 0$, $g_{13} = -1$; (4) $g_{43} = 0.5$, $g_{13} = -0.667$; (5) $g_{43} = 1$, $g_{13} = -0.333$; (6) $g_{43} = -1$, $g_{13} = -0.333$; (7) $g_{43} = 0.5$, $g_{13} = -0.667$; (8) $g_{43} = 0$, $g_{13} = -1$; (9) $g_{43} = 0.5$, $g_{13} = -1.33$; and (10) $g_{43} = 1$, $g_{13} = -1.67$.

Time courses are calculated by solving Eq. (7). As indicated in Eq. (8), derepression is stimulated by a step change in the repression factor f_r from 100 to 1 at $\tau = F_2 t = 0$. The initial condition is given by Eq. (9).

Fig. 5. Dimensionless settling time as a function of g_{43} and g_{45}. (a) Repressor-controlled systems with $g_{15} = -2$. (b) Activator-controlled systems with $g_{15} = 2$. The parameter estimates in Table 1, in addition to $L_{23} = -1$, apply to both panels. Settling time is defined as the dimensionless time τ required for enzyme to settle within 5% of its final steady-state value after derepression is initiated. Contours are equally spaced at 0.5 unit intervals. The dotted lines mark discontinuities. Settling times greater than 5.0 are not shown in panel (a) and settling times greater than 3.0 are not shown in panel (b). Settling times are calculated for the region of parameter space bounded by $|g_{43}|_{max} = |g_{45}|_{max} = |g_{13}|_{max} = 2$ and where $g_{45} < h_{44}h_{55} / g_{54}$. The same qualitative results are obtained if the region of parameter space considered is instead bounded by $|g_{43}|_{max} = |g_{45}|_{max} = |g_{13}|_{max} = 4$ (not shown).

Fig. 6. Comparisons of systems on the basis of temporal responsiveness are insensitive to uncertainty in estimates of turnover numbers. Results are shown for activator-controlled systems. Parameter values are the same as in Fig. 5(b), except each point corresponds to a set of equivalent systems with randomly selected values for F_1 (= F_4), F_2 (= F_5), and F_3. In panel (a), sets of equivalent systems with inverse coupling or direct coupling are considered; in panel (b), sets of equivalent systems with inverse coupling or uncoupling are considered; and in panel (c), sets of equivalent systems with direct coupling or uncoupling are considered. On the x-axis of each panel, each point indicates a minimal settling time, the fastest settling time found for a system of the type

indicated by the x-axis label among equivalent systems that share the point-specific turnover numbers. On the y-axis of each panel, each point indicates the ratio of minimal settling times found for the two types of systems being compared. Thus, in panel (a), $x = t^{(I)}_{min}$ and $y = t^{(I)}_{min} / t^{(D)}_{min}$; in panel (b), $x = t^{(I)}_{min}$ and $y = t^{(I)}_{min} / t^{(U)}$; and in panel (c), $x = t^{(D)}_{min}$ and $y = t^{(D)}_{min} / t^{(U)}$, where $t^{(I)}_{min}$ is the minimal settling time among a set of equivalent inversely coupled systems, $t^{(D)}_{min}$ is the minimal settling time among a set of equivalent directly coupled systems, and $t^{(U)}$ is the settling time of all equivalent uncoupled systems. A ratio less than 1 indicates that $t_{min}^{(I)} < t_{min}^{(D)}$ in panel (a), $t^{(I)}_{min} < t^{(U)}$ in panel (b), and $t^{(D)}_{min} < t^{(U)}$ in panel (c). The sampling of ratios is interpreted statistically by calculating a moving median of ratios[65], indicated by the curve in each panel. The moving median is based on a window size of 50 points.

Table 1. Parameter estimates[a].

Parameter	Estimate	Comment
g_{21} or g_{54}	1	Rate of protein synthesis is proportional to amount of mRNA
g_{32}	1	Rate of end-product synthesis is proportional to the amount of enzyme that catalyzes the reaction
g_{33}	-1	Biosynthetic pathways commonly involve feedback inhibition[66; 67; 68; 69], which is indicated *in vivo* for the *trp* system in *E. coli*[70; 71]. A value of -1 can be estimated based on the inhibition of anthranilate synthase activity *in vitro*[72].
h_{11} or h_{44}	1	Degradation is a first-order process[73]
h_{22} or h_{55}	1	Proteins are typically stable in bacteria[66]. Dilution of stable protein through exponential growth is a first-order process
h_{33}	0.5	Assuming Michaelis-Menten kinetics, the value of h_{33} lies between 0 and 1 with a value of 0.5 when the endogenous level of end-product equals the K_m for its consumption[24]. Based on analysis of the *trp* system in *E. coli*, [74; 75; 76; 77] we estimate that $h_{33} = 0.5$ for this system[b].
F_1 or F_4	1 min^{-1}	The half-life of anthranilate synthase mRNA, an effector mRNA in the *trp* system, is 43 s[78]. We assume that this half-life is typical for both regulator and effector mRNA.
F_2 or F_5	0.02 min^{-1}	We assume exponential growth and a doubling time of 40 min.
F_3	8 min^{-1}	Assuming a tryptophan composition of 54 μmol per g of dried cells, dry weight of 2.8 x 10^{-13} g per cell, a volume of 6.7 x 10^{-13} ml per cell, and a

		doubling time of 40 min, V_3 = 560 μM min^{-1}. We assume an endogenous concentration of tryptophan of X_{30} = 70 μM[76; 77].
f_r	100 or 1	For determining temporal responsiveness, we assume an initial repressed state that corresponds to f_r = 100. At the threshold of derepression, f_r = 1.

[a] Estimates for g_{21}, g_{32}, g_{54}, h_{11}, h_{22}, h_{44}, h_{55}, F_2, and F_5 are likely to hold for a variety of systems, especially in *E. coli* growing exponentially. Estimates for g_{33}, h_{33}, F_1, F_3, and F_4 are perhaps less general, being based on data specific for the tryptophan (*trp*) system in *E. coli*[2].

[b] In the *trp* system in *E. coli*, the pool of endogenous end-product, tryptophan, is depleted by the degradative activity of tryptophanase and the charging activity of tryptophan tRNA synthetase[74]. Because the apparent K_m for tryptophan in the formation of charged tRNA *in vitro*[75] (10 μM) is similar to the endogenous level of tryptophan[76; 77] (70 μM), we estimate that h_{33} = 0.5 for this system.

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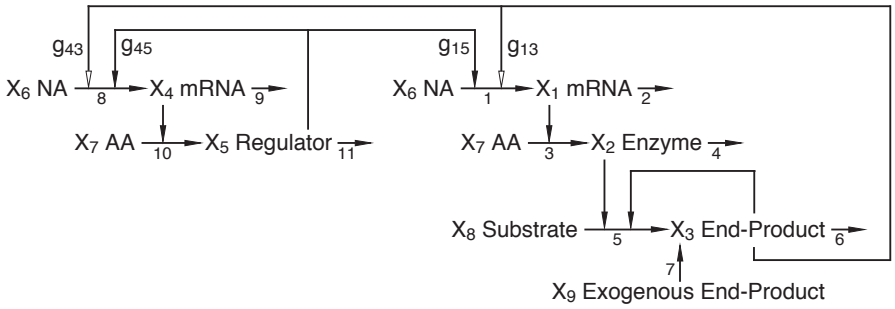
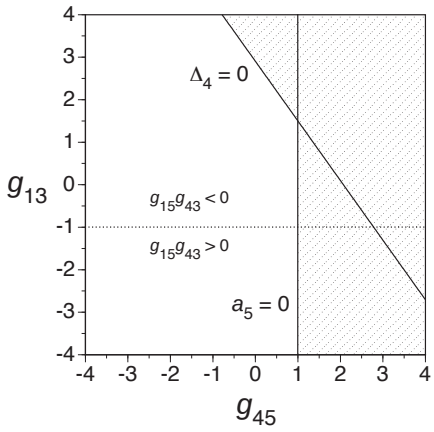
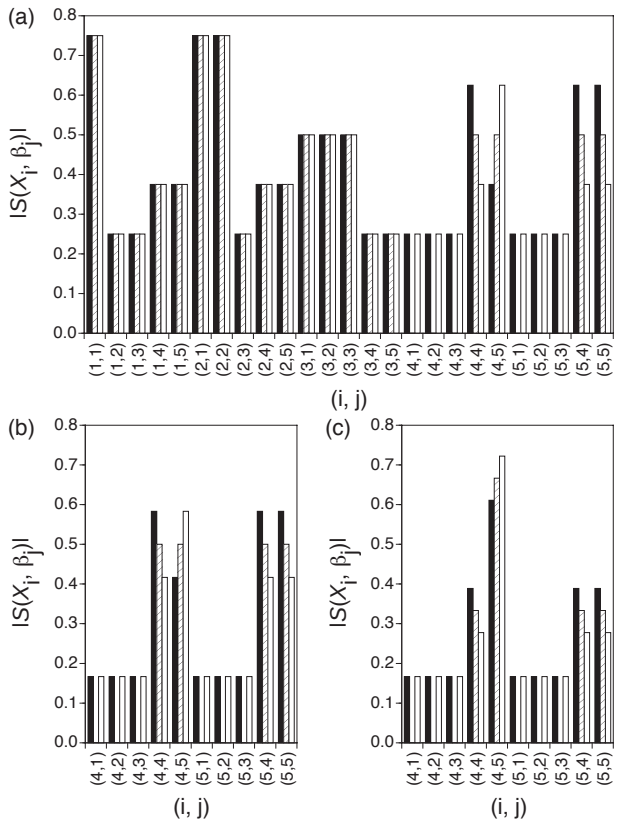
† Different formal definitions of a repressible system are possible. The definition that perhaps best corresponds to an experimental test for a repressible system is a system for which the gain L_{29} is negative. This gain, defined as $\partial \log X_2/\partial \log X_9$, characterizes the steady-state change in the level of enzyme X_2 caused by a change in the level of exogenous end-product X_9. The gain L_{29} is related to the gain L_{23} as follows: $L_{29} = -g_{39}L_{23}/(g_{32}L_{23}+g_{33}-h_{33})$. Thus, systems with the same gain L_{23} also have the same gain L_{29} provided these systems have the same values for g_{32}, g_{33}, g_{39}, and h_{33}, as we will require for internal equivalence. Given the parameter estimates noted in Table 1, $L_{29} < 0$ if $L_{23} < 0$ or if $L_{23} > (h_{33}-g_{33})/g_{32} > 0$. We consider only systems with $L_{23} < 0$. Systems with $L_{23} > 0$, although they can have $L_{29} < 0$, are not considered to be physiologically relevant[13].

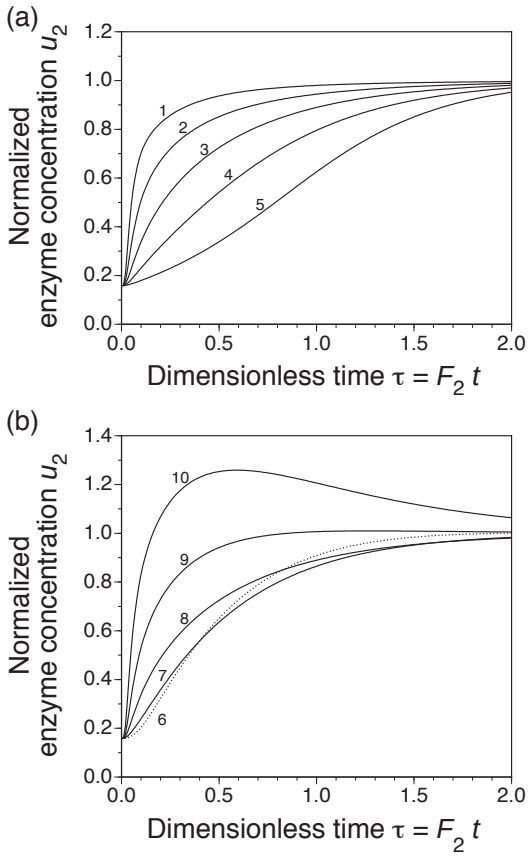
‡ Expansion of Eq. (5) yields $a_5 = K[1 - g_{32}L_{23}/(h_{33} - g_{33})](h_{44}h_{55} - g_{45}g_{54})$, where K is positive. It follows that $a_5 > 0$ if and only if $g_{45} < h_{44}h_{55}/g_{54}$. Note that $g_{32}L_{23}/(h_{33} - g_{33}) < 0$ if $g_{33} < 0$ (Table 1) and $L_{23} < 0$.

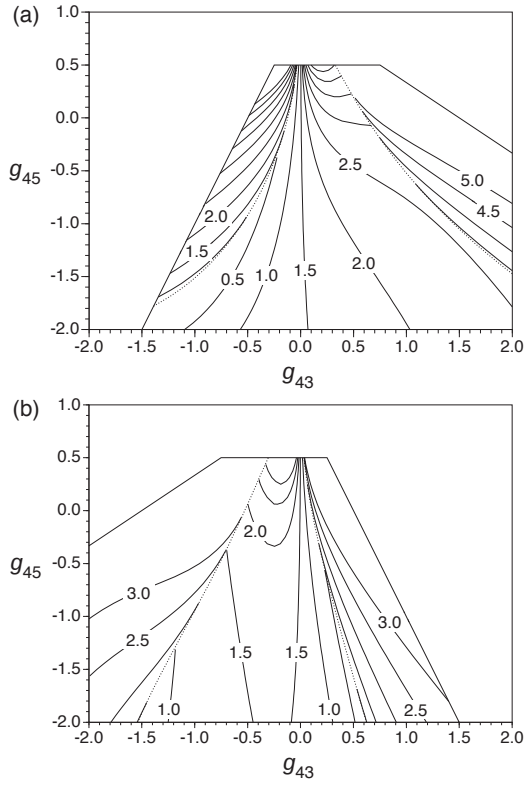
§ To consider the temporal responsiveness of different families of equivalent systems, we specified repressor- and activator-controlled reference systems with a range of values for the gain L_{23} (-1/4, -1/2, -3/4, -1, -5/4, -3/2, and -7/4). For each family, we considered a range of values for $\pm g_{15}$ (1/4, 1/2, 3/4, 1, 5/4, 3/2, 7/4, and 2).

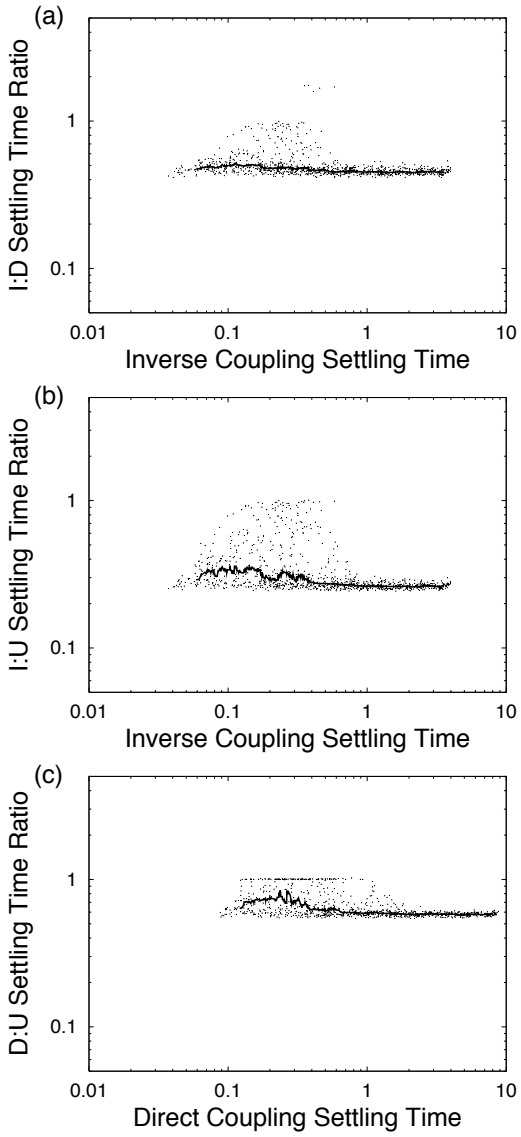

g43
g45
g15
g13
X6 NA
8
X4 mRNA
9
X6 NA
1
X1 mRNA
2
X7 AA
10
X5 Regulator
11
X7 AA
3
X2 Enzyme
4
X8 Substrate
5
X3 End-Product
6
7
X9 Exogenous End-Product


$\Delta_4 = 0$
$g_{15}g_{43} < 0$
$g_{15}g_{43} > 0$
$a_5 = 0$
g_{13}
g_{45}


(a)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
|S(Xi, βj)|
(1,1) (1,2) (1,3) (1,4) (1,5) (2,1) (2,2) (2,3) (2,4) (2,5) (3,1) (3,2) (3,3) (3,4) (3,5) (4,1) (4,2) (4,3) (4,4) (4,5) (5,1) (5,2) (5,3) (5,4) (5,5)
(i, j)
(b)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
|S(Xi, βj)|
(4,1) (4,2) (4,3) (4,4) (4,5) (5,1) (5,2) (5,3) (5,4) (5,5)
(i, j)
(c)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
|S(Xi, βj)|
(4,1) (4,2) (4,3) (4,4) (4,5) (5,1) (5,2) (5,3) (5,4) (5,5)
(i, j)


(a)
Normalized enzyme concentration u_2
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
4
5
0.0
0.5
1.0
1.5
2.0
Dimensionless time $\tau = F_2 t$
(b)
Normalized enzyme concentration u_2
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10
9
8
7
6
0.0
0.5
1.0
1.5
2.0
Dimensionless time $\tau = F_2 t$


(a)
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
g_{45}
2.5
5.0
4.5
2.0
1.5
0.5
1.0
1.5
2.0
-2.0 -1.5 -1.0 -0.5 0.0 0.5 1.0 1.5 2.0
g_{43}
(b)
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
g_{45}
2.0
3.0
2.5
1.5
1.5
3.0
1.0
1.0
2.5
-2.0 -1.5 -1.0 -0.5 0.0 0.5 1.0 1.5 2.0
g_{43}


(a)
I:D Settling Time Ratio
1
0.1
0.01
0.1
1
10
Inverse Coupling Settling Time
(b)
I:U Settling Time Ratio
1
0.1
0.01
0.1
1
10
Inverse Coupling Settling Time
(c)
D:U Settling Time Ratio
1
0.1
0.01
0.1
1
10
Direct Coupling Settling Time

SUPPLEMENTARY MATERIAL

Design Principles for Regulator Gene Expression in a Repressible Gene Circuit

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Graphical definitions of the gains L_{23} and L_{53}

The gains L_{23} and L_{53} are defined graphically in Fig. 7. Equation (1) predicts that the steady-state levels of effector and regulator gene expression will, in log-log coordinates, vary linearly with changes in the concentration of intracellular end-product. A system's gain L_{23} corresponds to the slope of the inclined portion of a plot of the type shown in Fig. 7(a). This slope is always negative for the circuits that we consider. (As mentioned in the main text, some circuits with a repressible phenotype may have $L_{23} > 0$ but we do not consider these circuits.) A system's gain L_{53} corresponds to the slope of the inclined portion of a plot of the type illustrated in Fig. 7(b). The gain L_{53} is negative for a directly coupled system, zero for an uncoupled system, and positive for an inversely coupled system.

Relationship between g_{13}, g_{15}, g_{43}, and g_{45} and systemic properties

The kinetic orders that characterize transcriptional control (g_{13}, g_{15}, g_{43}, and g_{45}) are the regulatory parameters of Eq. (1). In our comparisons, we vary the values of these kinetic orders to consider systems of different types and alternative systems within the same class. As discussed in the main text, given a reference system, the reference system and all systems considered to be equivalent to it can be represented in a three-dimensional parameter space, the coordinates of which are any three of the four regulatory parameters. The parameter space of g_{15}, g_{43}, and g_{45} and its features are illustrated in Fig. 8. Each position in this space corresponds to a system with the non-regulatory parameter values of the reference system and a unique set of regulatory parameter values. In this case, the value of the fourth regulatory parameter, g_{13}, is given by the following expression, which is derived from Eq. (2):

$$g_{13} = L_{23}h_{11}h_{22}/g_{21} - g_{15}g_{43}/(h_{44}h_{55}/g_{54} - g_{45}) \tag{11}$$

Interestingly, as this equation indicates, the value of g_{13}, which characterizes the influence of end-product on synthesis of effector mRNA, need not be negative, as one might expect, for effector gene expression to be repressible, i.e., g_{13} can be positive (and L_{23} can be negative) if the quantity $g_{15}g_{43}$ is negative and large enough in magnitude. Note that, as discussed in the main text, the denominator $h_{44}h_{55}/g_{54} - g_{45}$ in Eq. (11) is necessarily positive for a system with a stable steady state.

Useful alternative forms of Eq. (1)

Comparisons of gene circuits based on the criteria of stability and temporal responsiveness are facilitated by rewriting Eq. (1) as follows:

$$\begin{aligned}
du_1/dt &= F_1(u_6^{g_{16}}u_3^{g_{13}}u_5^{g_{15}} &&- u_1^{h_{11}})\\
du_2/dt &= F_2(u_7^{g_{27}}u_1^{g_{21}} &&- u_2^{h_{22}})\\
du_3/dt &= F_3(u_8^{g_{38}}u_9^{g_{39}}u_2^{g_{32}}u_3^{g_{33}} &&- u_3^{h_{33}})\\
du_4/dt &= F_4(u_6^{g_{46}}u_3^{g_{43}}u_5^{g_{45}} &&- u_4^{h_{44}})\\
du_5/dt &= F_5(u_7^{g_{57}}u_4^{g_{54}} &&- u_5^{h_{55}})
\end{aligned} \tag{12}$$

Here, $u_i = X_i/X_{i0}$ for $i = 1, \ldots, 9$ and $F_j = V_j/X_{j0}$ for $j = 1, \ldots, 5$, where X_{i0} is the steady-state value of X_i at the threshold of repression (Fig. 7) and $V_j = V_{+j} = V_{-j}$ is the steady-state

flux through the pool characterized by X_j. Each turnover number F_j has units consistent with the units of time t. Equation (7) in the main text is derived from Eq. (12), in part, by introducing dimensionless time $\tau = F_2 t$ and the dimensionless parameters $\epsilon = F_2/F_1$, $\mu = F_1/F_3$, and $f_r = (X_8/X_{80})^{g_{38}}(X_9/X_{90})^{g_{39}}$.

The linearization of Eq. (12), which is considered in comparisons based on the criterion of stability, can be written as $d\mathbf{x}/dt = \mathbf{L}\mathbf{x}$, where $\mathbf{x} = (x_1, \ldots, x_5)^T$, $x_i = X_i/X_{i0} - 1$ for $i = 1, \ldots, 5$, and

$$\mathbf{L} = \begin{pmatrix} -F_1 h_{11} & 0 & F_1 g_{13} & 0 & F_1 g_{15} \\ F_2 g_{21} & -F_2 h_{22} & 0 & 0 & 0 \\ 0 & F_3 g_{32} & -F_3(h_{33} - g_{33}) & 0 & 0 \\ 0 & 0 & F_4 g_{43} & -F_4 h_{44} & F_4 g_{45} \\ 0 & 0 & 0 & F_5 g_{54} & -F_5 h_{55} \end{pmatrix} \tag{13}$$

The characteristic polynomial of this matrix, $p(\lambda)$, is given in Eq. (5) of the main text.

Comparisons of gene circuits based on the criterion of robustness require that we calculate sensitivities, which are derived from steady-state expressions for concentrations and fluxes. The steady-state form of Eq. (1) can be written as $\mathbf{A}\mathbf{y} = \mathbf{b} + \mathbf{i}$, where $\mathbf{i}$ is a vector involving the independent concentrations X_6, X_7, X_8, and X_9, $\mathbf{b}$ is a vector defined as $(b_1, \ldots, b_5)^T$ in which each $b_i = \log \beta_i / \log \alpha_i$, $\mathbf{y}$ is a vector defined as $(y_1, \ldots, y_5)^T$ in which each $y_i = \log X_i$, and $\mathbf{A}$ is a matrix, given as

$$\mathbf{A} = \begin{pmatrix} -h_{11} & 0 & g_{13} & 0 & g_{15} \\ g_{21} & -h_{22} & 0 & 0 & 0 \\ 0 & g_{32} & g_{33} - h_{33} & 0 & 0 \\ 0 & 0 & g_{43} & -h_{44} & g_{45} \\ 0 & 0 & 0 & g_{54} & -h_{55} \end{pmatrix} \tag{14}$$

Each sensitivity $S(X_i, \beta_j)$ is identical with the element in the ith row and jth column of the inverse matrix $\mathbf{M} = \mathbf{A}^{-1}$. Note that the matrix $\mathbf{A}$ is invertible if $\det \mathbf{A} = g_{15}g_{21}g_{32}g_{43}g_{54} - (h_{44}h_{55} - g_{45}g_{54})[h_{11}h_{22}(h_{33} - g_{33}) - g_{13}g_{21}g_{32}] \neq 0$. As mentioned in the main text, other sensitivities are related to sensitivities of the form $S(X_i, \beta_j)$ (Savageau and Sorribas, 1989). For example, $S(X_i, \alpha_j) = -S(X_i, \beta_j)$, $S(X_i, g_{jk}) = g_{jk} S(X_i, \alpha_j) y_k$, and $S(X_i, h_{jk}) = h_{jk} S(X_i, \beta_j) y_k$.

The linkage coefficient

The relative stability of equivalent systems depends on just two of the four regulatory parameters (g_{13} and g_{45}), because the coefficients of the characteristic polynomial $p(\lambda)$ (Eq. (5)) are functions of g_{13}, g_{45}, and quantities that each have the same value for any two equivalent systems. This result follows from the constraints of equivalence, Eq. (5), and the following expression for the product of g_{15} and g_{43}, which is called the linkage coefficient (Savageau, 1985):

$$g_{15}g_{43} = (L_{23}h_{11}h_{22}/g_{21} - g_{13})(h_{44}h_{55}/g_{54} - g_{45}) \tag{15}$$

Equation (15) is obtained from Eq. (2). The dependence of the linkage coefficient on g_{13} and g_{45} is illustrated in Fig. 9. Thus, the coefficients of $p(\lambda)$ and the quantities in the necessary and sufficient conditions for stability ($a_1 > 0$, $a_3 > 0$, $a_5 > 0$, $\Delta_2 > 0$, and $\Delta_4 > 0$) depend on only two parameters that are potentially different for equivalent systems, g_{13} and g_{45}. Note that systems with different forms of coupling can have the same linkage coefficient.

The two critical stability conditions

As mentioned in the main text, destabilization of a system's steady state occurs when either of two necessary conditions for stability is violated (Frazer and Duncan, 1929). These two critical necessary conditions for stability are used to define a system's margin of stability. For systems described by Eq. (1), the conditions are $\Delta_4 > 0$ and $a_5 > 0$. In general, given a system with a characteristic monic polynomial $p(\lambda)$ of degree n with coefficients $a_1, \ldots, a_n$ as in Eq. (5) where $n = 5$, $a_n \Delta_{n-1} = 0$ everywhere on the boundary of stability that partitions systems with stable and unstable steady states in the coefficient space $(a_1, \ldots, a_n)$. The result that $a_n \Delta_{n-1} = 0$ can be obtained from 1) the proof of Fam (1977) that every point on the boundary of stability corresponds to a polynomial having at least one root with zero real part, 2) the root-coefficient relation $a_n = (-1)^n(\lambda_1 \lambda_2 \cdots \lambda_n)$, which indicates that a_n is zero if one or more roots of $p(\lambda)$ is zero, and 3) Orlando's formula $\Delta_{n-1} = (-1)^{\frac{n(n-1)}{2}} \Pi_{i<j\leq n}(\lambda_i + \lambda_j)$ (Gantmacher, 1959; Orlando, 1912), which indicates that Δ_{n-1} is zero if the sum of at least one pair of roots of $p(\lambda)$ is zero, as is the case if the polynomial has a pair of conjugate pure imaginary roots. In contrast, within the region of stability, which is contractible (Fam, 1977), $a_n > 0$ and $\Delta_{n-1} > 0$ (Gantmacher, 1959; Liénard and Chipart, 1914). Thus, because the zeros of a polynomial are continuous functions of the polynomial's coefficients (Marden, 1966), any continous change in a coefficient that leads to destabilization of a system's steady state (i.e., the emergence of a root of $p(\lambda)$ that has non-negative real part) must first occur when a_n and/or Δ_{n-1} equals zero. For systems described by Eq. (1), the same holds for changes in the regulatory parameters g_{13} and g_{45}, because the coefficients of $p(\lambda)$ (Eq. (5)) vary continuously with changes in these parameters, as can be confirmed.

The divergence and unstable oscillation curves

From the above considerations, it follows that the region of (g_{13}, g_{45})-parameter space in which systems with stable steady states are represented is bounded by two curves: the divergence curve along which $a_5 = 0$ and the unstable oscillation curve along which $\Delta_4 = 0$. The divergence curve, which is so called because points on this curve correspond to a zero eigenvalue, is the straight line along which $g_{45} = h_{44}h_{55}/g_{54}$. The unstable oscillation curve, which is so called because points on this curve correspond to a pair of conjugate pure imaginary eigenvalues, is an algebraic curve of order three along which $\sum_{i=0}^{3} \sum_{j=0}^{3-i} C_{ij} g_{13}^i g_{45}^j = 0$. In this formula, which is obtained from Eq. (5) and the expression for Δ_4, each C_{ij} is a function of quantities that are the same for equivalent systems, as can be confirmed.

The relative responsiveness of repressor-controlled systems is robust

Results similar to those presented in Fig. 6 of the main text are shown in Fig. 10. Recall that Fig. 6 shows that comparisons of activator-controlled circuits on the basis of temporal responsiveness are insensitive to uncertainty in estimates of turnover numbers. Comparisons of repressor-controlled circuits are likewise insensitive to uncertainty in estimates of turnover numbers (Fig. 10).

Graphical illustration of restrictions that derive from Eq. (4) on the form of coupling in systems with $|L_{23}| \geq |L_{23}^{\star}|$

The limitations of Eq. (4) on a system's form of coupling, which are indicated by Eq. (10) in the main text, are illustrated graphically in Fig. 11, which is interpreted as follows. Each panel shows a cross-section of the space (g_{15}, g_{43}, g_{45}) that represents a family of equivalent systems (Fig. 8). The value of g_{15} is invariant throughout each cross-section. Panels (a)–(c), on the left, indicate systems with repressor control of effector gene expression ($g_{15} < 0$), whereas panels (d)–(f), on the right, indicate systems with activator control of effector gene expression ($g_{15} > 0$). The magnitude of g_{15} is the same for all panels. The top panels ((a) and (d)), middle panels ((b) and (e)), and bottom panels ((c) and (f)) correspond to systems with high gain ($|L_{23}| > |L_{23}^{\star}|$), intermediate gain ($|L_{23}| = |L_{23}^{\star}|$), and low gain ($|L_{23}| < |L_{23}^{\star}|$), respectively. Each shaded region represents a set of systems with $g_{45} < h_{44}h_{55}/g_{54}$ and values for the kinetic orders g_{13}, g_{15}, g_{43}, and g_{45} that satisfy Eq. (4). For these systems, the signs of g_{43} and L_{53} are the same (Eq. (3)). Thus, a system represented in a shaded region has direct coupling if $g_{43} < 0$, uncoupling if $g_{43} = 0$, or inverse coupling if $g_{43} > 0$. We can now inspect Fig. 11 to confirm the following results. A repressor-controlled system with high gain is limited to inverse coupling (Fig. 11(a)), a repressor-controlled system with intermediate gain is limited to inverse coupling or uncoupling (Fig. 11(b)), an activator-controlled system with high gain is limited to direct coupling (Fig. 11(d)), and an activator-controlled system with intermediate gain is limited to direct coupling or uncoupling (Fig. 11(e)). Only in a repressor- or activator-controlled system with low gain do direct coupling, uncoupling, and inverse coupling each have the potential to be physically realized (Figs. 11(c,f)).

Analysis of ratios of sensitivities

The numerical results from comparisons on the basis of robustness discused in the main text are supplemented by considering ratios of sensitivities, which are given in Tables 2–4. These ratios are given for two arbitrary but equivalent systems: a reference system, whose unique parameter values are indicated by primes, and an alternative system. A ratio is interpreted by comparing its magnitude to 1. Because a sensitivity with a small magnitude indicates robustness, a ratio with magnitude greater than 1 indicates that the steady state of the reference system is more robust, whereas a ratio with magnitude less than 1 indicates that the steady state of the alternative system is more robust. The ratios in Table 2, on which we will focus, involve sensitivities of the form $S(V_3, p)$, where V_3 is the steady-state flux through the end-product pool and p is any of the rate constants or kinetic orders in Eq. (1). The results obtained by considering these ratios are qualitatively the same as those obtained by considering the full set of ratios, which are given in Tables 3 and 4.

The ratios in Table 2 either apply in general (column 2) or in special cases (columns 3 and 4). Each ratio in column 2 reduces to the corresponding ratio in column 3 if the following constraints are satisfied: $g_{13} = g'_{13}$, $g_{15} = g'_{15} (\neq 0)$, $g_{16} = g'_{16}$, and $g_{46} = g'_{46}$ (Special Case I). A consequence of these constraints is that the systems being compared have the same steady state at each and every level of repression. They also have the same gain L_{53} (from Eqs. (2) and (3)), which implies the same form of coupling. Each ratio in column 2 reduces to the corresponding ratio in column 4 if the following constraints are satisfied: $y_4 = y'_4$, $y_5 = y'_5$, $g_{15} = g'_{15} (\neq 0)$, $g_{16} = g'_{16}$, $g_{45} = g'_{45} < h_{44}h_{55}/g_{54}$, and $g_{46} = g'_{46}$ (Special Case II). A consequence of these constraints is that the systems

being compared have a similar or identical margin of stability, because they have the same mode and strength of autoregulation by the regulator protein (i.e., the same g_{45}).

Special Case I

Consideration of this case leads to the conclusion that negative autoregulation of regulator gene expression ($g_{45} < 0$) enhances robustness. From inspection of column 3 of Table 2, we find that each of the 29 ratios in Special Case I is less than or equal to 1 in magnitude when $g'_{45} > g_{45} \geq 0$, because $\delta = (h_{44}h_{55}/g_{54} - g'_{45})(h_{44}h_{55}/g_{54} - g_{45})^{-1} < 1$ and $g_{45}/g'_{45} < 1$ under this condition. Given that the choice of reference system considered in Table 2 is arbitrary, we conclude that there is a system with $g_{45} = 0$ that is more robust than any equivalent system with $g_{45} > 0$. Inspection of the ratios in column 3 of Table 2 also reveals that, when $g_{45} < g'_{45} = 0$, 28 of the 29 ratios are less than or equal to 1 in magnitude, with the magnitudes of these 28 ratios decreasing as g_{45} decreases. Thus, we conclude that there is a system with $g_{45} < 0$ that is more robust than any equivalent system with $g_{45} = 0$. The more negative the value of g_{45}, the greater the difference in robustness, regardless of the form of coupling.

Special Case II

We are motivated to consider this case, in which we focus on systems that have the same value for g_{45}, because of the results obtained above. Consideration of this case leads to the conclusion that directly coupled, uncoupled, and inversely coupled systems can be found that have a (derepressed) steady state with essentially the same robustness. As indicated in column 4 in Table 2, at least 27 of the 29 ratios equal 1 in magnitude in Special Case II. Because this result is independent of the values chosen for g_{43} and g'_{43}, we conclude that two systems with different forms of coupling (i.e., different signs for g_{43}) need not differ appreciably with respect to robustness. Recall that the signs of g_{43} and L_{53} are the same for systems with $g_{45} < h_{44}h_{55}/g_{54}$ (Eq. (3)), and thus, the sign of g_{43} indicates the form of coupling. The conclusion reached above is further supported by the observation that the ratio for the sensitivity of V_3 with respect to g_{43} can be made to have a magnitude of 1 by setting $g_{43} = -g'_{43}$, leaving just one ratio with magnitude different from 1. One can also confirm that differences between two systems are reduced as g_{45} becomes more negative.

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Table 2
Sensitivities of an arbitrary system relative to those of an equivalent reference system[a]

	$S(V_3,p)/S'(V_3,p)$		
Parameter p	General case	Special case I[b]	Special case II[c]
α_1, α_2, α_3, β_1, β_2, β_3, g_{21}, g_{27}, g_{32}, g_{33}, g_{38}, g_{39}, h_{11}, h_{22} or h_{33}	1	1	1
g_{16}	g_{16}/g'_{16}	1	1
α_4, α_5, β_4, β_5 or g_{57}	$(g_{15}/g'_{15})\delta$	δ	1
g_{46}	$(g_{46}/g'_{46})(g_{15}/g'_{15})\delta$	δ	1
g_{54} or h_{44}	$(y_4/y'_4)(g_{15}/g'_{15})\delta$	δ	1[d]
h_{55}	$(y_5/y'_5)(g_{15}/g'_{15})\delta$	δ	1[d]
g_{13}	g_{13}/g'_{13}	1	g_{13}/g'_{13}
g_{15}	$(y_5/y'_5)g_{15}/g'_{15}$	1	1[d]
g_{43}	$(g_{43}/g'_{43})(g_{15}/g'_{15})\delta$	1	g_{43}/g'_{43}
g_{45}	$(y_5/y'_5)(g_{45}/g'_{45})(g_{15}/g'_{15})\delta$	$(g_{45}/g'_{45})\delta$	1[d]

[a] Each entry is a ratio of the form $S(V_3,p)/S'(V_3,p)$: $S(V_3,p)$ is the sensitivity of an arbitrary system and $S'(V_3,p)$ is the sensitivity of an equivalent reference system. The ratio δ is defined as $(h_{44}h_{55}/g_{54}-g'_{45})/(h_{44}h_{55}/g_{54}-g_{45})$. Primed quantities are characteristic of the reference system, whereas unprimed quantities are characteristic of the alternative system or are common to both systems by equivalence.

[b] $g_{15}=g'_{15}\neq 0$, $g_{16}=g'_{16}$, $g_{46}=g'_{46}$, and $g_{13}=g'_{13}$. The systems being compared have the same form of coupling.

[c] $y_4=y'_4$, $y_5=y'_5$, $g_{15}=g'_{15}\neq 0$, $g_{16}=g'_{16}$, $g_{46}=g'_{46}$, and $g_{45}=g'_{45}\neq h_{44}h_{55}/g_{45}$. The systems being compared have the same mode and strength of autoregulation.

[d] This ratio can differ from 1 in magnitude away from the derepressed steady state $(X_{10},\ldots,X_{50})$, because in general, X_4 and X_5 are the same between equivalent systems only at the threshold of repression (Fig. 7(b)).

Table 3
Ratios of sensitivities of concentrations and fluxes to changes in rate constants[a]

Rate constant	Concentration or flux	
	X_1, X_2, X_3, V_1, V_2 or V_3	X_4, X_5, V_4 or V_5
α_1, α_2, α_3, β_1, β_2 or β_3	1	L_{53}/L'_{53}
α_4[b], β_4[b], α_5[c] or β_5[c]	$(g_{15}/g'_{15})\delta$	$\rho_1\delta$ or $\rho_2\delta$

[a] Each entry represents a ratio of the form $S(X_i,\alpha_j)/S'(X_i,\alpha_j)$, $S(X_i,\beta_j)/S'(X_i,\beta_j)$, $S(V_i,\alpha_j)/S'(V_i,\alpha_j)$, or $S(V_i,\beta_j)/S'(V_i,\beta_j)$, where the variable X_i or V_i is indicated by the column heading, and the parameter α_j or β_j is indicated by the row heading. The ratio δ is defined in Table 2. The ratios ρ_1 and ρ_2 are defined as follows: $\rho_1 = (A - g_{13})/(A - g'_{13})$ and $\rho_2 = [g_{15}g_{43} + g_{45}(A - g_{13})]/[g'_{15}g'_{43} + g'_{45}(A - g'_{13})]$, where $A = h_{11}h_{22}(h_{33} - g_{33})/(g_{21}g_{32})$.
[b] For X_4, X_5 and V_5, the entry is $\rho_1\delta$; for V_4, the entry is $\rho_2\delta$.
[c] For X_4, V_4 and V_5, the entry is $\rho_2\delta$; for X_5, the entry is $\rho_1\delta$.

Table 4
Ratios of sensitivities of concentrations and fluxes to changes in kinetic orders[a]

Kinetic order	Concentration or flux	
	X_1, X_2, X_3, V_1, V_2 or V_3	X_4, X_5, V_4 or V_5
g_{13}	g_{13}/g'_{13}	$(g_{13}/g'_{13})(g_{43}/g'_{43})\delta$
g_{15}	$(g_{15}/g'_{15})(y_5/y'_5)$	$(g_{15}/g'_{15})(y_5/y'_5)(g_{43}/g'_{43})\delta$
g_{16}	g_{16}/g'_{16}	$(g_{16}/g'_{16})(g_{43}/g'_{43})\delta$
h_{11}, g_{21}, g_{27}, h_{22}, g_{32}, g_{33}, g_{38}, g_{39} or h_{33}	1	$(g_{43}/g'_{43})\delta$
g_{43}	$(g_{43}/g'_{43})(g_{15}/g'_{15})\delta$	$(g_{43}/g'_{43})\rho_1\delta$
g_{45}	$(g_{45}/g'_{45})(y_5/y'_5)(g_{15}g'_{15})\delta$	$(g_{45}/g'_{45})(y_5/y'_5)\rho_1\delta$
g_{46}	$(g_{46}/g'_{46})(g_{15}/g'_{15})\delta$	$(g_{46})(g'_{46})\rho_1\delta$
h_{44}[b]	$(y_4/y'_4)(g_{15}/g'_{15})\delta$	$(y_4/y'_4)\rho_1\delta$ or $(y_4/y'_4)\rho_2\delta$
g_{54}[c]	$(y_4/y'_4)(g_{15}/g'_{15})\delta$	$(y_4/y'_4)\rho_1\delta$ or $(y_4/y'_4)\rho_2\delta$
g_{57}[d]	$(g_{15}/g'_{15})\delta$	$\rho_1\delta$ or $\rho_2\delta$
h_{55}[e]	$(y_5/y'_5)(g_{15}/g'_{15})\delta$	$(y_5/y'_5)\rho_1\delta$ or $(y_5,y'_5)\rho_2\delta$

[a] Each entry represents a ratio of the form $S(X_i,g_{jk})/S'(X_i,g_{jk})$, $S(X_i,h_{jk})/S'(X_i,h_{jk})$, $S(V_i,g_{jk})/S'(V_i,g_{jk})$, or $S(V_i,h_{jk})/S'(V_i,h_{jk})$, where the variable X_i or V_i is indicated by the column heading, and the parameter g_{jk} or h_{jk} is indicated by the row heading. The ratios δ, ρ_1, and ρ_2 are defined in Tables 2 and 3.
[b] For X_4, X_5, and V_5, the entry is $(y_4/y'_4)\rho_1\delta$; for V_4, the entry is $(y_4/y'_4)\rho_2\delta$.
[c] For X_4 and V_4, the entry is $(y_4/y'_4)\rho_2\delta$; for X_5 and V_5, the entry is $(y_4/y'_4)\rho_1\delta$.
[d] For X_4, and V_4, the entry is $\rho_2\delta$; for X_5 and V_5, the entry is $\rho_1\delta$.
[e] For X_4, V_4 and V_5, the entry is $(y_5/y'_5)\rho_2\delta$; for X_5, the entry is $(y_5/y'_5)\rho_1\delta$.

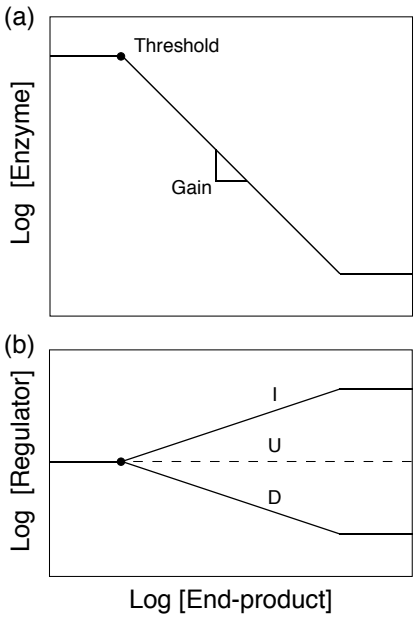

Fig. 7. Expression characteristics. The inclined portions of these log-log plots describe the steady-state input-output behavior of a system. (a) The effector expression characteristic. The level of effector protein (X_2) is shown as a function of the level of intracellular metabolic end-product (X_3). The inclined portion indicates the range of end-product concentration over which regulation takes place. The slope, which is negative for a repressible system, is quantified by the gain $L_{23} = \partial \log X_2 / \partial \log X_3$ (see Eq. (2)). The lower and upper plateaus indicate fully repressed and derepressed levels of effector expression. The steady state at the threshold of repression ($X_{10}, \ldots, X_{50}$) is indicated by a filled circle. (b) The regulator expression characteristic. The vertical axis indicates the level of regulator protein (X_5). A plot is shown for each of three cases: a system with direct coupling (D), a system with uncoupling (U), and a system with inverse coupling (I). The slope over the regulatable range of end-product concentration is quantified by the gain $L_{53} = \partial \log X_5 / \partial \log X_3$ (see Eq. (3)). Direct coupling is indicated when $L_{53} < 0$, uncoupling is indicated when $L_{53} = 0$, and inverse coupling is indicated when $L_{53} > 0$.

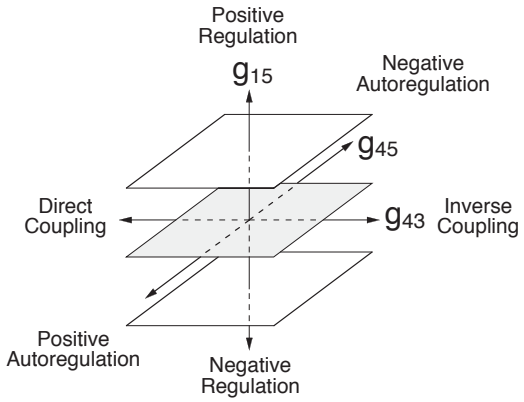

Fig. 8. The parameter space of g_{15}, g_{43}, and g_{45}. The entire family of systems that are equivalent to a particular reference system can be represented within this three-dimensional parameter space. The sign of g_{15} indicates the mode of effector gene regulation: negative for repressor control ($g_{15} < 0$) or postive for activator control ($g_{15} > 0$). Equivalent systems have the same mode of effector gene regulation and so are all represented together in either the upper or lower region of parameter space. The sign of g_{45} indicates the mode of regulator gene autoregulation, which can differ from the mode of effector gene regulation: negative for repressor control ($g_{45} < 0$) or postive for activator control ($g_{45} > 0$). If the regulator protein has no influence on its own synthesis, then $g_{45} = 0$. As discussed in the main text (see Eq. (3)), the signs of g_{43} and L_{53} are the same if $g_{45} < h_{44}h_{55}/g_{54}$, which is required for stability. Thus, $g_{43} < 0$ indicates direct coupling, $g_{43} = 0$ indicates uncoupling, and $g_{43} > 0$ indicates inverse coupling (Fig. 7(b)).

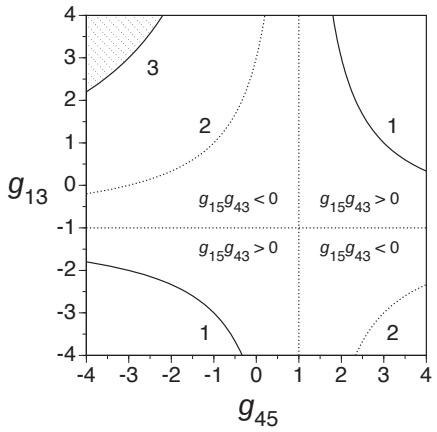

Fig. 9. Equivalent systems represented in the parameter space of g_{13} and g_{45}. In this space, the linkage coefficient, $g_{15}g_{43}$, is invariant along a hyperbola such as the one labeled (1), (2), or (3): (1) $g_{15}g_{43} = 4$, (2) $g_{15}g_{43} = -4$, (3) $g_{15}g_{43} = -16$. The dotted lines represent asymptotes, which are given by the equations $g_{45} = h_{44}h_{55}/g_{54}$ and $g_{13} = L_{23}h_{11}h_{22}/g_{21}$. These asymptotes divide the space into rectangular regions where the linkage coefficient is negative or positive. Uncoupled systems, which have $g_{15}g_{43} = 0$, are represented on the horizontal asymptote. If we assume $|g_{13}|_{\max} = |g_{15}|_{\max} = |g_{43}|_{\max} = |g_{45}|_{\max} = 4$, then realizable systems (i.e., systems that satisfy the constraints of Eq. (4)) are represented everywhere in this panel except the shaded region in the upper left corner.

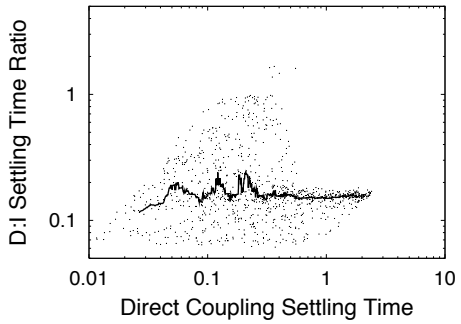

Fig. 10. Effect of uncertainty in turnover numbers on analysis of the temporal responsiveness of repressor-controlled systems. In this figure, $x = t_{\min}^{(\mathrm{D})}$ and $y = t_{\min}^{(\mathrm{D})}/t_{\min}^{(\mathrm{I})}$. The moving median is also shown, as described in Fig. 6. Inspection of this plot is sufficient to determine that the temporal responsiveness analysis is robust to uncertainty in turnover numbers, because for repressor-controlled systems, the minimal settling time for inversely coupled systems is always larger than the settling time for uncoupled systems, as illustrated in Fig. 5. Parameter values are the same as for Fig. 5(a), except turnover numbers are randomly selected (see Fig. 6).

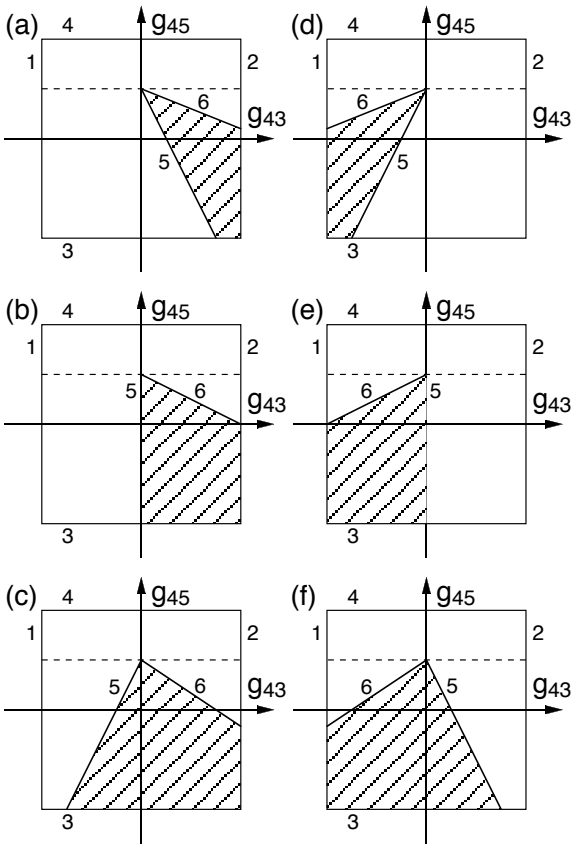

Fig. 11. Realizability of systems as a function of gain L_{23} and mode of regulation. Each panel is a representation of equivalent systems in the parameter space of g_{43} and g_{45}; the value of $|g_{15}|$ is the same for all systems. In each panel, the broken line marks the boundary of instability along which $a_5 = 0$. Points below this line represent systems with direct coupling if $g_{43} < 0$, uncoupling if $g_{43} = 0$, or inverse coupling if $g_{43} > 0$ (Fig. 3). Points within the shaded regions represent physically realizable systems, i.e., systems with kinetic orders that satisfy the constraints of Eq. (4). Lower and upper bounds on kinetic orders are indicated by lines along which (1) $g_{43} = -|g_{43}|_{\max}$, (2) $g_{43} = |g_{43}|_{\max}$, (3) $g_{45} = -|g_{45}|_{\max}$, (4) $g_{45} = |g_{45}|_{\max}$, (5) $g_{13} = -|g_{13}|_{\max}$, and (6) $g_{13} = |g_{13}|_{\max}$. The panels represent repressor-controlled (left) and activator-controlled (right) systems that have high (top), intermediate (middle), and low (bottom) gains: (a) $g_{15} < 0$ and $|L_{23}| > |L^{\star}_{23}|$, (b) $g_{15} < 0$ and $L_{23} = L^{\star}_{23}$, (c) $g_{15} < 0$ and $|L_{23}| < |L^{\star}_{23}|$, (d) $g_{15} > 0$ and $|L_{23}| > |L^{\star}_{23}|$, (e) $g_{15} > 0$ and $L_{23} = L^{\star}_{23}$, (f) $g_{15} > 0$ and $|L_{23}| < |L^{\star}_{23}|$ where $L^{\star}_{23} = |g_{13}|_{\max}[g_{21}/(h_{11}h_{22})]$.