

## REPORT

# The incoherent feed-forward loop can generate non-monotonic input functions for genes

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Gene regulation networks contain recurring circuit patterns called network motifs. One of the most common network motif is the incoherent type 1 feed-forward loop (I1-FFL), in which an activator controls both gene and repressor of that gene. This motif was shown to act as a pulse generator and response accelerator of gene expression. Here we consider an additional function of this motif: the I1-FFL can generate a non-monotonic dependence of gene expression on the input signal. Here, we study this experimentally in the galactose system of *Escherichia coli*, which is regulated by an I1-FFL. The promoter activity of two of the *gal* operons, *galETK* and *galP*, peaks at intermediate levels of the signal cAMP. We find that mutants in which the I1-FFL is disrupted lose this non-monotonic behavior, and instead display monotonic input functions. Theoretical analysis suggests that non-monotonic input functions can be achieved for a wide range of parameters by the I1-FFL. The models also suggest regimes where a monotonic input-function can occur, as observed in the *mglBAC* operon regulated by the same I1-FFL. The present study thus experimentally demonstrates how upstream circuitry can affect gene input functions and how an I1-FFL functions within its natural context in the cell.

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## Introduction

Transcription networks contain recurring circuits called network motifs (Shen-Orr *et al.*, 2002; Alon, 2007). A small number of network motif designs seem to appear again and again in the transcription networks of *Escherichia coli*, yeast and higher organisms (Lee *et al.*, 2002; Milo *et al.*, 2002; Odom *et al.*, 2004). Experiments and theory have outlined some of the key dynamic functions that can be carried out by each network motif (reviewed in Alon, 2007).

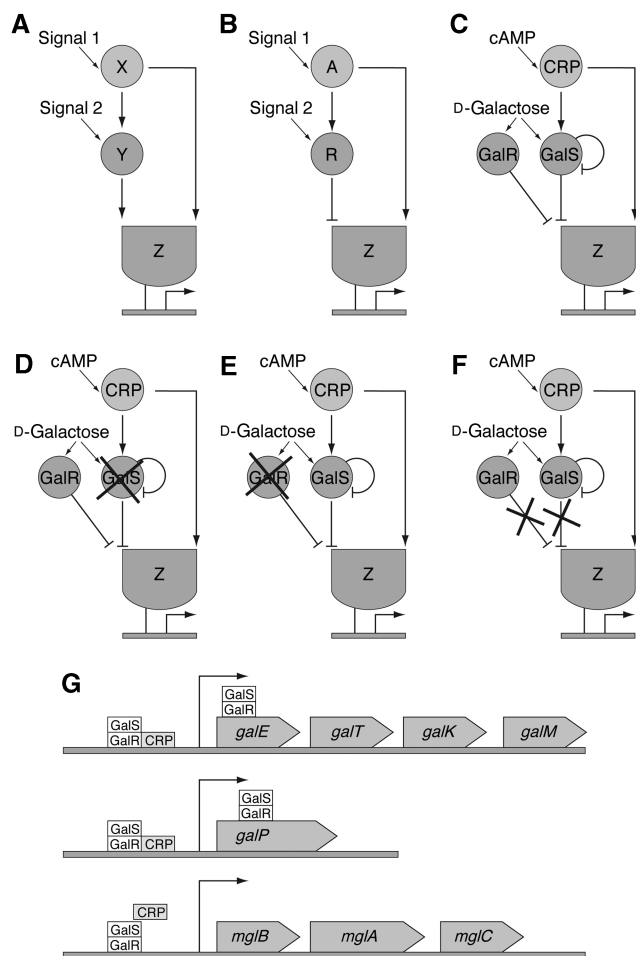
One of the most prevalent network motifs in transcription networks is the feed-forward loop (FFL) (Mangan and Alon, 2003; Eichenberger *et al.*, 2004; Alon, 2006). The FFL is made up of two transcription factors, *X* and *Y*, and a target gene *Z*. In the FFL, *X* regulates the promoter of the gene for *Y*, and both *X* and *Y* regulate the target promoter *Z*. Thus, the FFL has two

parallel paths: a direct path from *X* to the target gene and an indirect path through *Y* (see Box 1).

The FFL motif is further classified into eight subtypes based on the mode of regulation (activator or repressor) of its three interaction arrows (Mangan and Alon, 2003; Alon, 2007). It appears that in the transcription networks of *E. coli* and yeast, two of these eight FFL types are found much more commonly than the other six. These are the coherent type 1 FFL and the incoherent type 1 FFL (I1-FFL) (Box 1A and B).

In contrast to the coherent FFL, in which both regulatory paths have the same effect (activation), in the incoherent I1-FFL the two paths have opposite effects. In one path, *A* activates the target gene *Z*, but in the second path, *A* activates a repressor of *Z* (Box 1B). This design was shown theoretically and experimentally to have a speedup function, where *Z*

**Box 1 The incoherent type 1 feed-forward loop in the *gal* system**



An overview of the feed-forward loop (FFL) network motif in the *gal* system together with promoter structures and mutations used in this study.

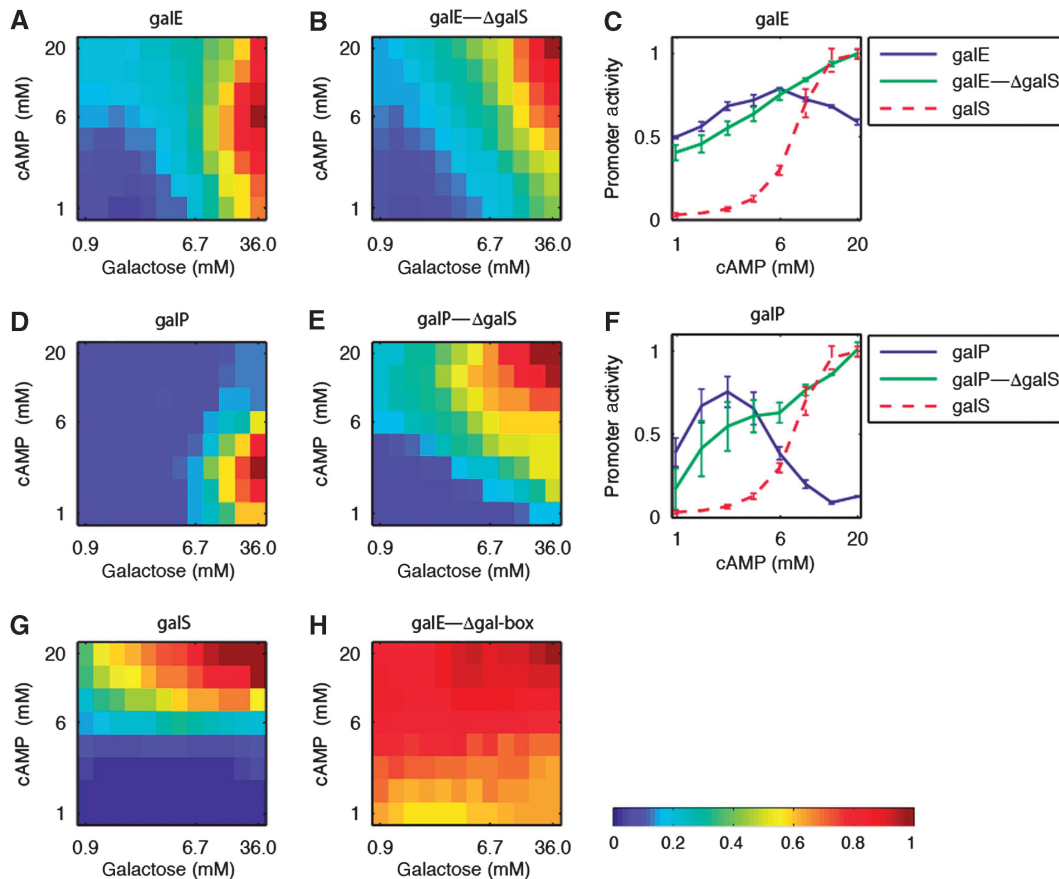
One of the most prevalent network motifs in transcription networks is the feed-forward loop. The FFL is made of two transcription factors, X and Y, and a target gene Z. In the FFL, X regulates the promoter of the gene for Y, and both X and Y regulate the target promoter Z. Thus, the FFL has two parallel paths: a direct path from X to the target gene, and an indirect path through Y. Two of the most common FFL circuits in the transcription networks of bacteria and yeasts are the coherent type 1 FFL and the incoherent type 1 FFL (I1-FFL). (A) Coherent type 1 FFL network motif has two activators. (B) The I1-FFL has an activator A that activates repressor R, and both regulate the target gene Z. (C) The *galE* upstream circuitry is in the form of an I1-FFL. It includes the repressor GalS that is activated by CRP to repress the target genes. GalR is a constitutive repressor that also represses the *gal* genes, but does not participate in the I1-FFL circuit. (D) The *galI1*-FFL was disrupted in this study by a *galS* deletion. (E) The *galI1*-FFL with a *galR* deletion (simple example of I1-FFL). (F) GalS/GalR binding sites on the *galE* promoter were deleted, generating a CRP-only regulated promoter. (G) Promoter structure of the studied genes (based on Ecocyc).

responds more rapidly to the input signal than it would have in the absence of the I1-FFL (Mangan *et al*, 2006).

In addition to this speedup feature, theoretical studies have predicted that the I1-FFL may be used to produce non-monotonic response, also called an amplitude filter or biphasic response (Basu *et al*, 2004, 2005; Ishihara *et al*, 2005). In this type of response, the output Z first increases with the input signal that activates A, but decreases with signal when the signal is high. This function was experimentally demonstrated by the construction of synthetic I1-FFL circuits using

well-characterized activators and repressors, resulting in a tunable non-monotonic response (Basu *et al*, 2005; Entus *et al*, 2007). Such theoretical and synthetic circuit studies focus on the I1-FFL in isolated systems. To demonstrate that the expected function is carried out *in vivo*, it is necessary to study the I1-FFL in its natural context where the circuit is wired into the full interaction networks of the cell.

Here, we study the function of the I1-FFL in such a natural system. We employ the I1-FFL in the *gal* system of *E. coli* (Weickert and Adhya, 1993), and ask whether it can generate a



**Figure 1** Non-monotonic input function in the *gal* system becomes monotonic when *galS* is deleted. (A, D) Non-monotonic two-dimensional input function of *galE* and *galP* in the wild-type background. The two axes correspond to the signals for the I1-FFL regulators: cAMP and galactose. (B, E) In a  $\Delta galS$  background, *galE* and *galP* input functions are monotonic with an AND-like response, typical for other sugar systems. (C, F) One-dimensional input function of the *gal* genes as a function of cAMP at saturating (36 mM) D-galactose. (G) Two-dimensional input function of *galS*. (H) A mutant of the *galE* promoter in which the binding sites for GalS and GalR repressors were deleted shows a monotonic response to cAMP with a high basal expression level. Promoter activity is the rate of GFP fluorescence accumulation per OD unit in exponential phase. The figure shows promoter activity normalized to its maximal value for each promoter.

non-monotonic input function *in vivo*. The *gal* system includes genes that utilize the sugar galactose (the *galETK* operon), and pumps that transport the sugar into the cell (*galP*, *mglB* genes) (Weickert and Adhya, 1993; Neidhart, 1996). The promoters of these genes serve as output (*Z*) promoters in I1-FFLs, in which A and R are the cAMP-responsive activator CRP and the galactose-responsive repressor GalS. A second repressor, GalR, is constitutive (Box 1C) (Mangan *et al*, 2006; Semsey *et al*, 2006). In a survey of the input functions of *E. coli* sugar systems, it was recently found that the activity of two of the *gal* promoters, *galE* and *galP*, peaked at intermediate levels of cAMP (Figure 1A and D), the input signal of A (Kaplan *et al*, 2008). This is in line with biochemical data that indicated that at high cAMP levels the *galE* promoter is repressed (Semsey *et al*, 2006).

Here, we find that disrupting the I1-FFL circuit in the *gal* system changes the input function from non-monotonic to monotonic. Thus, the amplitude filter feature depends on the I1-FFL architecture. We also present a simple model for the I1-FFL that shows that amplitude filtering is expected for a wide range of biochemical parameters. These results suggest that the amplitude filter function of the I1-FFL is a feature of

this gene circuit that appears in the natural context within the cell.

## Results

### Detailed mapping of gene input functions

To measure promoter activity, we used an automated assay based on fluorescent reporter strains (Kaplan *et al*, 2008). Reporter strains used in this study are from a comprehensive *E. coli* transcription reporter library (Zaslaver *et al*, 2006). Each strain in this library bears a low-copy plasmid with GFP under the control of a full-length copy of the promoter of interest. The GFP variant (GFP mut 2; Cormack *et al*, 1996) in these strains becomes fluorescent within minutes and yields a bright signal. In this study, we used the reporters for the *galETK*, *galP*, *galS* and *mglB* promoters.

To map the input function of each promoter, cultures of reporter strains were grown in 96-well plates. The plates contained 8 levels of cAMP in the rows and 12 levels of D-galactose in the columns, producing 96 different combinations of the two inducers. A robotic system prepared the medium

and set up the plates. Each plate was seeded with one of the reporter strains and transferred to an automated shaker incubator. Each experiment also included a control plate that was seeded with a promoterless reporter strain that was used for fluorescence background subtraction. The plates were periodically transferred by the robot into a multiwell fluorimeter, which allowed measurement of GFP fluorescence and cell optic density (OD) at a resolution of 8 min.

Cells were grown on glucose minimal medium to minimize endogenous production of cAMP and achieve growth rate that is independent of the inducer levels, a condition that enabled accurate mapping of the input function as described (Setty *et al*, 2003; Kaplan *et al*, 2008). The range of added cAMP concentration (1–20 mM) led to promoter activities that span the physiological activation range of known CRP-regulated sugar-utilizing genes (Baker *et al*, 2001; Setty *et al*, 2003; Kaplan *et al*, 2008), as well as of a synthetic promoter regulated only by CRP (Supplementary Figure 2). A control strain ( $\Delta$ *CyaA*; Holland *et al*, 1988; Kuhlman *et al*, 2007) that lacks the ability to produce endogenous cAMP shows results that are very similar to those of the wild-type strain, demonstrating that the effects of endogenous cAMP production are negligible under the present conditions (see Supplementary Figure 1).

Promoter activity was defined as the rate of GFP production per OD unit,  $x = d\text{GFP}/dt/\text{OD}$  (see Materials and methods). The input function was calculated by averaging the promoter activity over a one-cell-generation window in exponential phase (~6 h from initial 1:600 inoculation) where promoter activity was constant to a good approximation. The input functions are reproducible to within less than 10% mean error in repetitive experiments.

### The *galE* and *galP* promoter shows maximal activity at intermediate cAMP levels

In a recent study, we have found that in the presence of galactose, the *galE* and *galP* promoter input functions peak at intermediate cAMP levels (Figure 1A and D; see also Kaplan *et al*, 2008). Their activity increases with cAMP levels up to an intermediate external concentration and then decreases, decaying to about 1/4 of the maximal level for the *galP* promoter and to about 2/3 for the *galE* promoter. The *galS* promoter activity increases with cAMP levels, consistent with the known transcriptional activation of *galS* by CRP (Figure 1G) in this I1-FFL. As the range of cAMP concentrations needed for activation of the *galS* promoter overlaps with the concentrations at which the promoter activity of *galE* and *galP* decreases (Figure 1C and F), one may hypothesize that the induction of the GalS repressor leads to the observed non-monotonic pattern.

### Deletion of the *galS* gene results in monotonically increasing input function

We studied the possible contribution of the I1-FFL to the non-monotonic behavior by mapping the input function of *galE* and *galP* in an isogenic strain that had a genomic deletion of the *galS* gene (Box 1D) (Baba *et al*, 2006; see Materials and methods). We find that in the  $\Delta$ *galS* strain, *galE* and *galP* show monotonic input functions that increase with cAMP levels

(Figure 1B and E). At low cAMP levels, this function is very similar to that of the wild-type strain and rises with cAMP levels at about the same rate as in the wild-type strain. However, whereas the wild type peaks and then decreases at high cAMP levels, the  $\Delta$ *galS* strain shows no peak and saturates at high cAMP levels. Thus, in a strain in which the I1-FFL is disrupted by deletion of the *galS* repressor, non-monotonic input function changes to a monotonic one.

Note that sensitivity to galactose is maintained in the  $\Delta$ *galS* strain (Figure 1B and E), owing to the presence of the second *gal* repressor, GalR. GalR is a constitutively expressed repressor, and, in contrast to GalS, is not part of the I1-FFL, as it is not regulated by CRP.

We also disrupted the I1-FFL by another means: we deleted the repressor binding sites from the target *galE* promoter (Box 1F) while keeping the repressor gene intact. We find that the input function of a reporter strain in which the GalS/GalR binding sites in the *galE* promoter were deleted is monotonic (Figure 1H). This experiment indicates that the non-monotonic behavior is due to Gal repressor action and not due to a hypothetical direct repression by CRP at high cAMP levels.

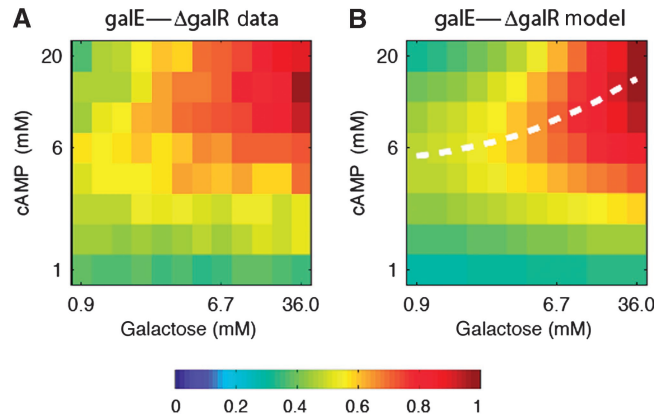
### Deletion of *galR* maintains the non-monotonic shape of the input function

We also studied the input function of the *galE* promoter in an isogenic strain deleted for the *galR* gene (Box 1E). In this strain, the *galS* I1-FFL remains intact and the constitutive *galR* repressor is missing. We find that the  $\Delta$ *galR* strain preserves a non-monotonic shape for the *galE* input function, with expression that peaks at intermediate levels of cAMP (Figure 2A). The position of this peak changes with galactose concentrations: the peak position rises from 6 mM cAMP at low galactose to about 20 mM cAMP at high galactose levels (Figure 2A).

We also find that the input function has a higher basal level than in the wild-type strain: the *galR* deletion strain shows expression of the *galE* promoter (even in the absence of galactose) that is at least two-fold higher than its most induced activity in the presence of GalR. This is consistent with earlier studies that indicated that GalR is the major repressor for *galE* and is responsible for most of its galactose sensitivity (Geanacopoulos and Adhya, 1997; Semsey *et al*, 2007).

### The I1-FFL generates a monotonic input function in the *mglB* promoter

We also studied the input function of the *mglB* promoter, which controls the expression of the galactose ABC transporter genes and is regulated by the gal I1-FFL. We find that the input function of the *mglB* promoter is monotonic (Supplementary Figure 3A), rising continuously with cAMP levels. Thus, in addition to the non-monotonicity found in the *galE* and *galP* promoters, we find that the I1-FFL can also result in monotonic input functions. The monotonic *mglB* input function is maintained also in a  $\Delta$ *galS* strain in which the I1-FFL is disrupted (Supplementary Figure 3B). The difference between the *galE/galP* and the *mglB* input functions suggests that the I1-FFL can be designed to show either monotonic or



**Figure 2** Input function of *galE* in a  $\Delta galR$  background shows non-monotonic behavior to cAMP. **(A)** Experimental measurements. **(B)** A simple model of I1-FFL (see equations (1–3)) shows a good fit to the measured input function with parameters  $K_1=5$  mM,  $K_2=5$  mM and  $k_g=5$  mM. The position of the peak (concentration of cAMP at which promoter activity is maximal) increases with the concentration of galactose (white dashed line).

non-monotonic behavior, depending on biochemical parameters. To address the effects of biochemical parameters on the gene input function, we now turn to a mathematical analysis of the I1-FFL.

### A simple model for I1-FFL input functions

To model a simple I1-FFL and compare it to experimental data, we study the *galE* input function in a strain with deletion of *galR* (see Figure 2A). This strain provides a simple *in vivo* model for the I1-FFL motif as it contains a single repressor (GalS), rather than the two repressors (GalS and GalR) of the wild-type strain. To make a model that captures the essentials if not the full details of this system, we use the standard modeling approach based on equilibrium binding of transcription factors to their sites on a promoter, as reviewed in Bintu *et al* (2005) and Entus *et al* (2007). Consider an activator that binds a promoter with the dissociation constant  $k_A$  and a repressor that binds with the dissociation constant  $k_R$ . The promoter is transcriptionally active when the activator but not the repressor binds. The promoter activity is then described by a ratio of two polynomials corresponding to the partition function of the binding process, whose variables are the concentrations of an active activator  $A$  and an active repressor  $R$ :

$$P = \frac{A/k_A}{1 + A/k_A + R/k_R + (A/k_A)(R/k_R)} \quad (1)$$

In the I1-FFL, the repressor level  $R$  increases with the activity of  $A$  (as CRP activates the *galS* gene). The repression activity of  $R$  is inhibited by the inducer (galactose), with half-effect at inducer concentration of  $k_g$  (equation (2)). These effects can be described most simply by the following equation that rises linearly with  $A$  and has a Michaelis-Menten-like effect of  $g$ :

$$R = \frac{A}{1 + g/k_g} \quad (2)$$

By plugging in the repressor level from equation (2) into equation (1) one obtains an expression for I1-FFL input function. This equation gives rise to non-monotonic behavior (equation (3)), because the denominator increases as a

function of  $A^2$ , whereas the numerator increases as a function of  $A$  and thus the function first rises and then decreases as a function of  $A$ :

$$P = \frac{A/k_A}{1 + A/k_A + A/(k_R(1 + g/k_g)) + A^2/(k_A k_R(1 + g/k_g))} \quad (3)$$

We find that this model provides a good fit to the data for the *galE* input function in the  $\Delta galR$  strain (Figure 2A and B).

The model also allows an analytical solution for the position of the peak in the input function. To find the level of  $A$  (i.e. the cAMP concentration) at which the promoter activity is maximal, one needs to find the value of  $A$  where the derivative  $dP/dA$  is zero. The solution  $A_{\max}$  rises with the dissociation constants and with the inducer level:

$$A_{\max} = \sqrt{k_A k_R} \sqrt{1 + g/k_g} \quad (4)$$

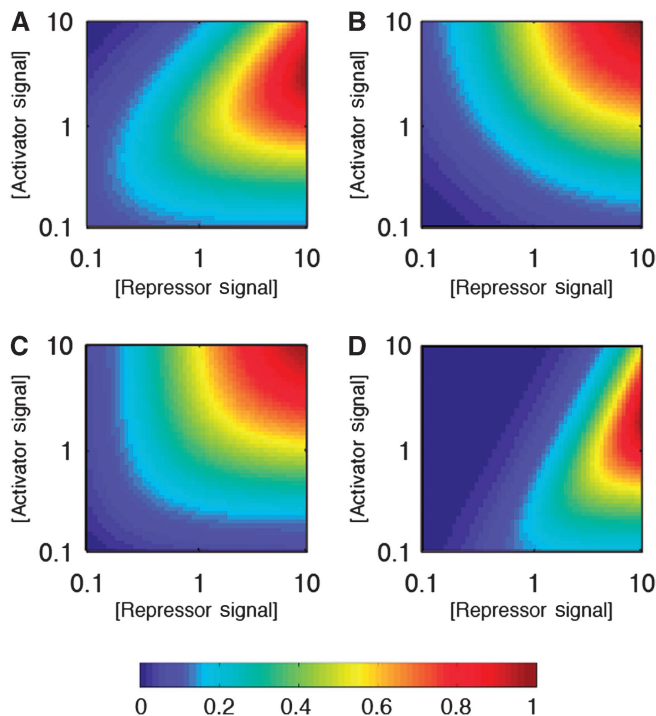
The position of the maximum is thus proportional to the geometric mean of the dissociation constants  $k_A$  and  $k_R$ . Differences in these effective binding constants may explain the difference in the observed peak position between the *galP* promoter (peak  $\sim 3$  mM cAMP) and the *galE* promoter (peak  $\sim 6$  mM cAMP) (Figure 1A and D). In addition, the peak position is expected to increase with the level of galactose  $g$ . This agrees with the observed shift in the peak position to higher cAMP levels with increasing galactose levels (dashed white line in Figure 2B) in the experiments presented above.

The I1-FFL of the *galE* promoter in a  $\Delta galR$  strain might also include a self-inhibitory effect of the GalS on its own promoter. However, a theoretical analysis shows that this should not have a significant effect on the above analysis (see Supplementary Figure 4).

### Theoretical analysis suggests necessary conditions for non-monotonic input functions

Finally, we consider additional possible effects that can shape the input function of the I1-FFL. The aim is to find conditions in which the I1-FFL can generate non-monotonic input functions for a wide range of parameters. We also sought to





**Figure 3** Models of I1-FFL demonstrate conditions in which non-monotonic or monotonic input functions are achieved. **(A)** Non-monotonicity emerges in I1-FFL given that simultaneous binding of the activator and repressor is possible. **(B)** Elimination of the I1-FFL eliminates the non-monotonic behavior. **(C)** A monotonic input function is found in the case of exclusive binding of *A* and *R* when the cooperativity coefficients on both arms of the FFL are similar ( $h_1=1$ ,  $h_2=1$ ,  $h_3=1$ ). **(D)** A non-monotonic input function is found in the case of exclusive binding of *A* and *R* when the cooperativity of the indirect arm of the I1-FFL exceeds that of the direct arm (here,  $h_1=1$ ,  $h_2=2$ ,  $h_3=1$ ) (see Materials and methods for model description).

find conditions under which the I1-FFL shows monotonic functions.

We first add Hill-like kinetics to the model of the previous section, described by effective Hill coefficients  $h_1$  and  $h_2$ :

$$P = \frac{(A/k_A)^{h_1}}{1 + (A/k_A)^{h_1} + (R/k_R)^{h_2} + (A/k_A)^{h_1} (R/k_R)^{h_2}}$$

Here,  $h_1$  is the apparent Hill coefficient describing the cooperativity of *A* action and  $h_2$  is that for *R*. In the I1-FFL the activator *A* also activates the gene for *R*. Thus one has  $R \sim A^{h_3}$ , if the promoter of the *R* gene is far from saturation, where  $h_3$  is the apparent Hill coefficient for the action of *A* on the promoter of the gene for *R*. Non-monotonic behavior is always found in this case (Figure 3A), because when *A* activity is large, the last term in the denominator goes as  $A^{(h_1+h_3h_2)}$ , whereas the numerator goes as  $A^{h_1}$ . For positive Hill coefficients,  $h_1 + h_3h_2$  always exceeds  $h_1$ , and hence the promoter function decreases at high levels of *A* activity. Only if the promoter of the *R* gene saturates at low levels of *A* activity, before the quadratic term in the denominator is significantly larger than the other terms, will non-monotonic behavior be lost. Thus, when both factors *A* and *R* can bind the promoter simultaneously, non-monotonic behavior is obtained for a wide range of parameters.

An alternative promoter design is that binding of *A* and *R* cannot occur at the same time owing to steric hindrance. This has been suggested to apply to the *gal* promoters (Semsey *et al*,

2006), because upon binding to their two binding sites the repressors are assumed to form a loop that prevents the CRP from binding to the promoter. In this case, the promoter activity can be modeled without the interaction term in the denominator:

$$P = \frac{(A/k_A)^{h_1}}{1 + (A/k_A)^{h_1} + (R/k_R)^{h_2}}$$

Here, as above,  $A \sim R^{h_3}$  in the case of a non-saturated promoter for gene *R*. Non-monotonic behavior is found when  $h_2h_3 > h_1$ , because the denominator outweighs the numerator at high values of *A* activity (Figure 3C, D). For example, in the case of the *gal* system, the present data for the *galS* promoter suggest that  $h_3 \sim 2$  and that this promoter does not seem to saturate at the measured activity levels of *A* (Figure 1G). The *galE* promoter seems to have  $h_1 \sim 1$ , and thus  $h_2h_3 > h_1$  and non-monotonic behavior is found (Figure 1C).

In summary, when both activator and repressor can simultaneously bind the promoter, non-monotonic input functions only require that the *R* promoter remains unsaturated at high levels of *A* activity. When *A* and *R* cannot bind the promoter simultaneously, non-monotonic behavior requires, in addition to an unsaturated *R* promoter, that the combined cooperativity of the indirect arm of the I1-FFL—the product of the cooperativity of *A* on the *R* promoter and *R* on the *Z* promoter—exceeds that of the direct arm, given by the cooperativity of *A* on the *Z* promoter (Figure 3).

To explain the monotonic behavior found in the *mglB* promoter, the present theory offers several possibilities. Monotonic behavior can be found if  $k_R$ , which reflects the threshold of the repressor, is high enough (e.g. weak affinity to its site in the promoter). This is because the repressor then has only a limited effect within its physiological concentration range, and the peak level is pushed to very high  $A$  levels beyond the physiological range. An alternative scenario for monotonic behavior occurs if  $A$  and  $R$  bind with similar cooperativity but interfere with each others' binding (Figure 3C). Support for this possibility lies in the structure of the *mglB* promoter in which partial overlap between the repressors and activator binding sites is observed (Box 1G).

## Discussion

This study experimentally demonstrated that the I1-FFL in the *gal* system of *E. coli* generates non-monotonic input functions in the *galE* and *galP* promoters. Disruptions of the I1-FFL, either by deleting the repressor or by deleting its binding sites in the downstream promoter, abolished the non-monotonic shape of these input functions and resulted instead in monotonically increasing input functions. We also demonstrate that the I1-FFL can generate a monotonic input function in the *mglB* promoter regulated by the same network motif.

As recently suggested by mapping a range of different input functions, non-monotonic input functions are particular to the galactose system and not to most other sugar systems studied. These other sugar systems are regulated by coherent FFLs and not I1-FFLs. The non-monotonic input functions in the galactose system might be related to the dual role of galactose in *E. coli* metabolism both as a carbon source and as a component of the cell wall (Weickert and Adhya, 1993). Under this constraint, when the cells are severely starved for glucose, they might reduce galactose breakdown and redirect it to structural purposes.

The present theoretical analysis suggests that non-monotonic input functions can occur for a wide range of parameters of the I1-FFL, provided that the overall cooperativity of the repressive path exceeds that of the direct path. These conditions seem to apply to the case of the *galE* and *galP* promoters, and may not apply to the case of the monotonic *mglB* promoter. The theoretical analysis explains why the position of the peak of the input function moves to higher cAMP levels with increasing galactose levels. It also suggests how different promoters can have different peak positions, due to differential affinities of the regulators to each promoter.

The finding that non-monotonicity (amplitude filtering) occurs for a wide range of parameters agrees with synthetic engineering studies, in which amplitude filters were readily obtained by wiring activators and repressors together in an I1-FFL pattern, with no need to tune parameters (Basu *et al*, 2005; Entus *et al*, 2007). The present study tested this function in a natural gene circuit, which is embedded in the regulatory and metabolic networks of the cell. The finding that the I1-FFL shows its expected features *in vivo* is in line with the view that network motifs can act as elementary circuit modules even within the cellular context.

This study adds experimental evidence for a new role of the I1-FFL, showing that it can generate complex input functions of two input signals, one of which peaks at intermediate values. This adds to previously studied functions, in which the I1-FFL can generate a pulse of expression and speed up the response time of the system. Future experimental studies can explore whether this motif carries out a similar function in other gene systems. More generally, it is of interest to experimentally explore the functions of network motifs in different systems and different organisms (Lahav *et al*, 2007; Yakoby *et al*, 2007; Temme *et al*, 2008). This may lead to the establishment of a dictionary of information processing functions that these elementary circuits can perform (Guido *et al*, 2006; Murphy *et al*, 2007).

## Materials and methods

### Reporter strains

GFP reporter plasmids for *galE*, *galP*, *mglB* and *galS* in the wild-type background (MG1655) are from the fluorescent reporter library given in detail in Zaslaver *et al* (2006). A mutated reporter plasmid for *galE* was obtained by deleting the GalS/GalR binding sites (Keseler *et al*, 2005; Karp *et al*, 2007) in the promoter region on the plasmid (Baseclear Labservices). The deleted sequences are tgtgtaaacgattccac around -60 bp from initiation of transcription and tatgagagttcgttaccgggttagcggttaca at around +50 bp. Isogenic  $\Delta galS$  and  $\Delta galR$  strains were obtained by transducing the deletions from the Keio knockout collection derived from the BW25113 strain (Baba *et al*, 2006) into MG1655 by P1 transduction. The genomic deletion was verified by PCR. Kanamycin resistance was eliminated from the deleted strain using FLP recombinase, as described (Datsenko and Wanner, 2000). We then transformed the  $\Delta galS$  and  $\Delta galR$  strains with the reporter plasmids for *galE*, *galP*, *mglB* and *galS*.

### Growth conditions and measurements

Reporter strains (five different reporter strains + promoterless control strain pUA66 in each experiment) were grown overnight in M9 minimal medium containing 0.4% glucose, 0.2% casamino acids and 50  $\mu$ g/ml kanamycin at 37°C. Using a robotic liquid handler (FreedomEvo, Tecan), 96-well plates were prepared with 150  $\mu$ l of M9 minimal medium containing 0.2% glucose, 0.05% casamino acids, 25  $\mu$ g/ml kanamycin and 96 different combinations of the system inducers (8 levels of serially diluted cAMP (1:1.5) and 12 levels of serially diluted D-galactose (1:1.4)). The wells were inoculated with the reporter strain at a 1:600 dilution from the overnight culture. This high dilution factor allowed a prolonged exponential phase. Wells were then covered with 100  $\mu$ l of mineral oil (Sigma) to prevent evaporation, a step that we previously found not to significantly affect aeration or growth (Ronen *et al*, 2002; Zaslaver *et al*, 2004), and transferred into an automated incubator. Cells were grown in an incubator with shaking (6 Hz) at 30°C for about 20 h. Every 8 min the plate was transferred by the robotic arm into a multiwell fluorimeter (Infinite F200, Tecan) that read OD (600 nm) and GFP fluorescence (535 nm). Plasmid copy number did not vary measurably over the growth conditions (Kaplan *et al*, 2008).

### Data analysis

Promoter activity for each well was calculated from the OD and GFP measurements after subtracting the OD and GFP backgrounds as described (Kaplan *et al*, 2008). GFP background was obtained for each well from the promoterless control strain U66. Promoter activity was calculated by computing the rate of accumulation of GFP per unit time divided by the OD (dGFP/dt/OD) (Ronen *et al*, 2002). The promoter activity was averaged over a window of 80 min ( $\sim 1$  cell cycle at exponential growth). Over this window, promoter activity varied less

than 20%. Each input function was normalized to its maximal level and smoothed with a median filter (medfilt2 of Matlab 7.0). Growth rate ( $dOD/dt/OD/\ln(2)$  in units of doublings/h) over this time window was very similar for all conditions ( $0.6 \pm 0.03$  doublings/h).

## Model input functions

The model input functions presented in Figure 3 were obtained using Matlab 7.1. A grid of equally spaced logarithmic scale axes from  $10^{-1}$  to  $10^1$  was used for both the activator inducer  $y$  (so that  $A$  activity is  $A=y$ ) and the inhibitor signal  $x$  of the repressor  $R$  so that  $R$  activity is inversely proportional to  $x$ . In the case in which the repressor is not regulated by the activator (no I1-FFL),  $R_0=K_{RX}/x$ . In the cases in which  $R$  is activated by  $A$  (I1-FFL),  $R$  is, for an unsaturated promoter design, proportional to  $A$  so that  $R_1=K_{RX}A/x$ . In the case in which  $R$  activation by  $A$  is unsaturated and cooperative, the repressor can be described as  $R_2=K_{RX}A^h/x$ . If the  $Z$  repressor allows simultaneous binding of activator and repressor with no I1-FFL (Figure 3A), one has  $P=A/(1+A+R_0+AR_0)$ . Simultaneous binding of activator and repressor with I1-FFL (Figure 3B) corresponds to  $P=A/(1+A+R_1+AR_1)$ . An I1-FFL with exclusive binding with similar cooperativity of activator and repressor (Figure 3C) is described by  $P=A/(1+A+R_1)$ . An I1-FFL with exclusive binding with cooperativity of the repressor binding ( $h=2$ ) (Figure 3D) is modeled by  $P=A/(1+A+R_1^2)$ . An I1-FFL with exclusive binding with cooperativity of the activator-repressor interaction using  $R_2$  (Figure 3E) is modeled by  $P=A/(1+A+R_2)$ .

## Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website ([www.nature.com/msb](http://www.nature.com/msb)).

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