Signaling architectures that transmit unidirectional information

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Abstract

A signaling pathway transmits information from an upstream system to downstream systems, ideally unidirectionally. A key bottleneck to unidirectional transmission is retroactivity, which is the additional reaction flux that affects a system once its species interact with those of downstream systems. This raises the question of whether signaling pathways have developed specialized architectures that overcome retroactivity and transmit unidirectional signals. Here, we propose a general mathematical framework that provides an answer to this question. Using this framework, we analyze the ability of a variety of signaling architectures to transmit signals unidirectionally as key biological parameters are tuned. In particular, we find that single stage phosphorylation and phosphotransfer systems that transmit signals from a kinase show the following trade-off: either they impart a large retroactivity to their upstream system or they are significantly impacted by the retroactivity due to their downstream system. However, cascades of these architectures, which are highly represented in nature, can overcome this trade-off and thus enable unidirectional information transmission. By contrast, single and double phosphorylation cycles that transmit signals from a substrate impart a large retroactivity to their upstream system and are also unable to attenuate retroactivity due to their downstream system. Our findings identify signaling architectures that ensure unidirectional signal transmission and minimize crosstalk among multiple targets. Our results thus establish a way to decompose a signal transduction network into architectures that transmit information unidirectionally, while also providing a library of devices that can be used in synthetic biology to facilitate modular circuit design.

Author Summary

Although signaling pathways in cells are typically viewed as transmitting information unidirectionally between an upstream and downstream system, such a viewpoint is not accurate in general due to retroactivity. Retroactivity in the added reaction flux that changes the behavior of the upstream system because of the reactions its species participate in to transmit information to downstream processes. Large retroactivity effects are therefore a major bottleneck to unidirectional signal transmission. Thus, a framework that can identify signaling architectures that overcome retroactivity and transmit unidirectional signals (and those that do not) is required to accurately simplify and analyze signal transduction networks. In this work, we develop such a framework and analyze several signaling architectures to test for their ability to transmit unidirectional signals. We find that cascades of signaling cycles that transmit information via kinases are well-suited to unidirectional transmission. In contrast, signaling systems that transmit information via substrates are highly susceptible to effects of retroactivity. They are thus not well-suited to unidirectional signal transmission, which may explain their low frequency of occurrence in natural systems. Our results thus provide key insights into cellular signal transduction, as well as provide a library of devices for synthetic biology that could be used for unidirectional signaling.

1 Introduction

Cellular signal transduction is typically viewed as a unidirectional transmission of information via biochemical reactions from an upstream system to multiple downstream systems through signaling pathways [1]- [7]. However, without the

presence of specialized mechanisms, signal transmission via chemical reactions is not in general unidirectional. In fact, the chemical reactions that allow a signal to be transmitted from an upstream to downstream systems also affect the upstream system due to the resulting reaction flux. This flux is called retroactivity, which is one of the chief hurdles to one-way transmission of information [8]- [13]. Signaling pathways, typically composed of phosphorylation, dephosphorylation and phosphotransfer reactions, are highly conserved evolutionarily, such as the MAPK cascade [14] and two-component signaling systems [15]. Thus, the same pathways act between different upstream and downstream systems in different scenarios and organisms, facing different effects of retroactivity in different contexts. What then may allow signal transmission to be unidirectional in these different contexts? We hypothesize that, for ideal unidirectional signal transmission, signaling pathways must have specific architectures that overcome retroactivity. In particular, these architectures should impart a small retroactivity to the upstream system (called retroactivity to the input) and should not be affected by the retroactivity imparted to them by the downstream systems (retroactivity to the output).

Phosphorylation-dephosphorylation cycles, phosphotransfer reactions, and cascades of these are ubiquitous in both prokaryotic and eukaryotic signaling pathways, playing a major role in cell cycle progression, survival, growth, differentiation and apoptosis [1]- [7], [16]- [19]. Numerous studies have been conducted to analyze such systems, starting with milestone works by Stadtman and Chock [20], [21], [22] and Goldbeter et al. [23], [24], [25], which theoretically and experimentally analyzed phosphorylation cycles and cascades. These systems were further investigated by Kholdenko et al. [26], [27], [28] and Gomez-Uribe et al. [29], [30]. However, these studies considered signaling cycles in isolation, and thus did not investigate the effect of retroactivity. The effect of retroactivity on such systems was theoretically analyzed in the work by Ventura et al. [31], where retroactivity is treated as a "hidden feedback" to the upstream system. Experimental studies then confirmed the effects of retroactivity in signaling systems through *in vivo* experiments on the MAPK cascade [12], [13] and *in vitro* experiments on reconstituted covalent modification cycles [9], [11]. These studies clearly demonstrated that the effects of retroactivity on a signaling system manifest themselves in two ways. They cause a slow down of the temporal response of the signaling system's output to its input and lead to a change of the output's steady state.

In 2008, Del Vecchio et al. demonstrated theoretically that a single phosphorylation-dephosphorylation (PD) cycle with a slow input kinase can attenuate the effect of retroactivity to the output when the total substrate and phosphatase concentrations of the cycle are increased together [8]. Essentially, a sufficiently large phosphatase concentration along with relatively large kinetic rates of modification adjusts the cycle's internal dynamics very quickly with respect to a relatively slower input, making any retroactivity-induced delays negligible on the time scale of the signal being transmitted [32]. A similarly large concentration of total cycle's substrate ensures that the output signal is not attenuated with respect to the input signal and that the output's steady state is not significantly affected by the presence of downstream sites. These theoretical findings were later verified experimentally both *in vitro* [11] and *in vivo* [33]. Although a single PD cycle can attenuate the effect of retroactivity to the output, it is unfortunately unsuitable for unidirectional signal transmission. In fact, as the substrate concentration is increased, the PD cycle applies a large retroactivity to the input, causing the input signal to slow down. This was experimentally observed in [33]. The results of [34] further suggest that a cascade composed of two PD cycles and a phosphotransfer reaction could overcome both retroactivity to the input and retroactivity to the output. In [35], it was theoretically found that, for certain parameter conditions, a cascade of PD cycles could attenuate the upward (from downstream to upstream) propagation of disturbances applied downstream of the cascade. These results suggest that PD cycles, phosphotransfer reactions, and their combinations may be able to counteract retroactivity. Thus, signaling architectures composed of PD cycles and phosphotransfer reactions may be ideal candidates for allowing signal transmission to be unidirectional. However, to the best of the authors' knowledge, no attempt has been made to systematically characterize signaling architectures with respect to their ability to overcome the effects of retroactivity and therefore enable unidirectional signal transmission.

This work presents a generalized mathematical framework to identify and characterize signaling architectures that can transmit unidirectional signals. This framework is based on a reaction-rate ordinary differential equation (ODE) model for a general signaling system that operates on a fast timescale relative to its input. Such a model is valid for many signaling systems that transmit relatively slower signals, such as those from slowly varying "clock" proteins that operate on the timescale of the circadian rhythm [36], from proteins signaling nutrient deficiency [37], or from proteins whose concentration is regulated by transcriptional networks which operate on the slow timescale of gene expression [38]. Our framework provides expressions for retroactivity to the input and to the output as well as the input-output relationship of the signaling system. These expressions are given in terms of the reaction-rate parameters and protein concentrations. Based on these expressions, we analyze a number of signaling architectures composed of PD cycles and phosphotransfer

systems. For these architectures, we determine whether their total (modified and unmodified) protein concentrations can be tuned to simultaneously minimize retroactivity to the input and attenuate retroactivity to the output. We focus on total protein concentrations as a design parameter because these appear to be highly variable in natural systems and through the course of evolution, where they may have been optimized to improve systems' performance [39], [40]. Protein concentration is also an easily tunable quantity in synthetic genetic circuits. We thus identify signaling architectures where we can tune total protein concentrations to both minimize retroactivity to the input and attenuate retroactivity to the output, thus ensuring unidirectional signal transmission.

2 Results

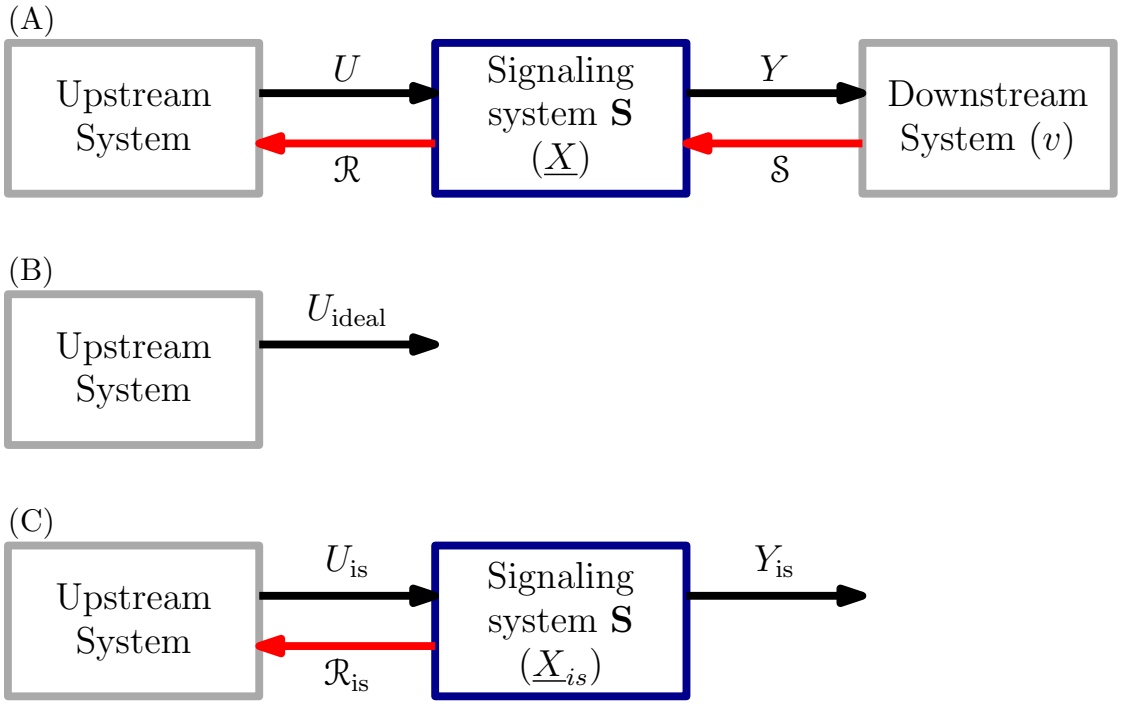


Fig 1. Interconnections between a signaling system S and its upstream and downstream systems, along with input, output and retroactivity signals. (A) Full system showing all interconnection signals: $U(t)$ is the input from the upstream system to the signaling system, with state variable vector $\underline{X}$. $Y(t)$ is the output of the signaling system, sent to the downstream system, whose state variable is v. $\mathcal{R}$ is the retroactivity signal from the signaling system to the upstream system (retroactivity to the input of **S**), and $\mathcal{S}$ is the retroactivity signal from the downstream system to the signaling system (retroactivity to the output of **S**). (B) Ideal input U_{ideal}: output of the upstream system in the absence of the signaling system ($\mathcal{R} = 0$). (C) Isolated output Y_{is}: output of the signaling system in the absence of the downstream system ($\mathcal{S} = 0$). $\underline{X}_{\text{is}}$ denotes the corresponding state of **S**.

In this section, we consider a general signaling system **S** with state-variable vector of protein concentrations $\underline{X}$ as shown in Fig. 1A. Each component of $\underline{X}$ represents the concentration of a species composing system **S**. This system **S** is connected between an upstream system from which it receives an input in the form of a protein with concentration U, and a downstream system to which it sends an output in the form of a protein with concentration Y. When the output protein reacts with the species of the downstream system, whose normalized concentrations are represented by state variable v, the resulting reaction flux changes the behavior of the upstream system. We represent this reaction flux as an additional input, $\mathcal{S}$, to the signaling system. Similarly, when the input protein from the upstream system reacts with the species of the signaling system, the resulting reaction flux changes the behavior of the upstream system. We represent this as an input, $\mathcal{R}$, to the upstream system. We call $\mathcal{R}$ the retroactivity to the input of **S** and $\mathcal{S}$ the retroactivity to the output of **S**, using the notation proposed in [8]. For system **S** to transmit a unidirectional signal, the effects of $\mathcal{R}$ on the upstream system and of $\mathcal{S}$ on the downstream system must be small. Retroactivity to the input $\mathcal{R}$ changes the input from

U_{ideal} to U, where U_{ideal} is shown in Fig. 1B. Thus, for the effect of $\mathcal{R}$ to be small, the difference between U and U_{ideal} must be small. Retroactivity to the output $\mathcal{S}$ changes the output from Y_{is} to Y, where Y_{is} is shown in Fig 1C, and for the effect of retroactivity to the output to be small, the difference between Y_{is} and Y must be small. An *ideal unidirectional signaling system* is therefore a system where the input U_{ideal} is transmitted from the upstream system to the signaling system without any change imparted by the latter, and the output Y_{is} of the signaling system is also transmitted to the downstream system without any change imparted to it by the downstream system. Based on this concept of ideal unidirectional signaling system, we then present the following definition of a signaling system that can transmit information unidirectionally. In order to give the following definition, we assume that the proteins (besides the input species) that compose signaling system **S** are constitutively produced and therefore their total concentrations (modified and unmodified) are constant. The vector of these total protein concentrations is denoted by $\underline{\Theta}$.

Definition 1. We will say that system **S** is a signaling system that can transmit unidirectional signals for all inputs $U \in [0, U_b]$, if $\underline{\Theta}$ can be chosen such that the following properties are satisfied:

(i) $\mathcal{R}$ is small: this is mathematically characterized by requiring that $|U_{\text{ideal}}(t) - U(t)|$ be small for all $U \in [0, U_b]$.

(ii) System **S** attenuates the effect of $\mathcal{S}$ on Y: this is mathematically characterized by requiring that $|Y_{\text{is}}(t) - Y(t)|$ be small for all $U \in [0, U_b]$.

(iii) Input-output relationship: $Y_{\text{is}}(t) \approx K U_{\text{is}}(t)^m$, for some $m \geq 1$, for some $K > 0$ and for all $U \in [0, U_b]$.

Note that Def. 1 specifies that the signaling system must impart a small retroactivity to its input (i) and attenuate retroactivity to its output (ii). In particular, it specifies that these properties should be satisfied for a full range of inputs and outputs, implying that these properties must be guaranteed by the features of the signaling system and cannot be enforced by tuning the amplitudes of inputs and/or outputs.

As an illustrative example of the effects of $\mathcal{R}$ and $\mathcal{S}$ on a signaling architecture, we consider a signaling system **S** composed of a single PD cycle [8], [11], [33]. The system is shown in Fig. 2A. It receives a slowly varying input signal U in the form of kinase concentration Z generated by an upstream system, and has as the output signal Y the concentration of X^*, which in this example is a transcription factor that binds to promoter sites in the downstream system. Kinase Z phosphorylates protein X to form X^*, which is dephosphorylated by phosphatase M back to X. The state variables $\underline{X}$ of **S** are the concentrations of the species in the cycle, that is, X, M, X^*, C_1, C_2, where C_1 and C_2 are the complexes formed by X and Z during phosphorylation, and by X^* and M during dephosphorylation, respectively. The state variable v of the downstream system is the normalized concentration of C, the complex formed by X^* and p (i.e., $v = \frac{C}{p_T}$ where p_T is the total concentration of the downstream promoters). This configuration, where a signaling system has as downstream system(s) gene expression processes, is common in many organisms as it is often the case that a transcription factor goes through some form of covalent modification before activating or repressing gene expression [41]. However, the downstream system could be any other system, such as another covalent modification process, which interacts with the output through a binding-unbinding reaction. We denote the total amount of cycle substrate by $X_T = X + X^* + C_1 + C_2 + C$ and the total amount of phosphatase by $M_T = M + C_2$.

According to Def. 1, we vary the total protein concentrations of the cycle, $\underline{\Theta} = [X_T, M_T]$, to investigate the ability of this system to transmit unidirectional signals. To this end, we consider two extreme cases: first, when the total substrate concentration X_T is low (simulation results in Figs. 2B, 2C); second, when it is high (simulation results in Figs. 2D, 2E). For both these cases, we change M_T proportionally to X_T. This is because, for large Michaelis-Menten constants, we have an input-output relationship with $m = 1$ and $K \approx \frac{k_1 K_{m2}}{k_2 K_{m1}} \frac{X_T}{M_T}$ (details in SI Section 5.2, eqn. (23)) as defined in Def. 1(iii). To maintain the same K for fair comparison between the two cases, we vary M_T proportionally with X_T. Here, K_{m1} and k_1 are the Michaelis-Menten constant and catalytic rate constant for the phosphorylation reaction, and K_{m2} and k_2 are the Michaelis-Menten constant and catalytic rate constant for the dephosphorylation reaction. These reactions are shown in eqns. (18) in SI Section 5.2. For the simulation results, we consider a sinusoidal input to see the dynamic response of the system to a time-varying signal. For these two cases then, we see from Fig. 2B that when X_T (and M_T) is low, $\mathcal{R}$ is small, i.e., $|U_{\text{ideal}}(t) - U(t)|$ is small (satisfying requirement (i) of Def. 1). This is because kinase Z must phosphorylate very little substrate X, and thus, the reaction flux due to phosphorylation to the upstream system is small. However, as seen in Fig. 2C, for low X_T, the signaling system is unable to attenuate $\mathcal{S}$. The difference $|X^*_{\text{is}} - X^*|$ is large, and requirement (ii) of Def. 1 is not satisfied for low X_T. This large retroactivity to the output is due to the reduction in the total substrate available for the cycle because of the sequestration of X^* by the promoter sites in the downstream system.

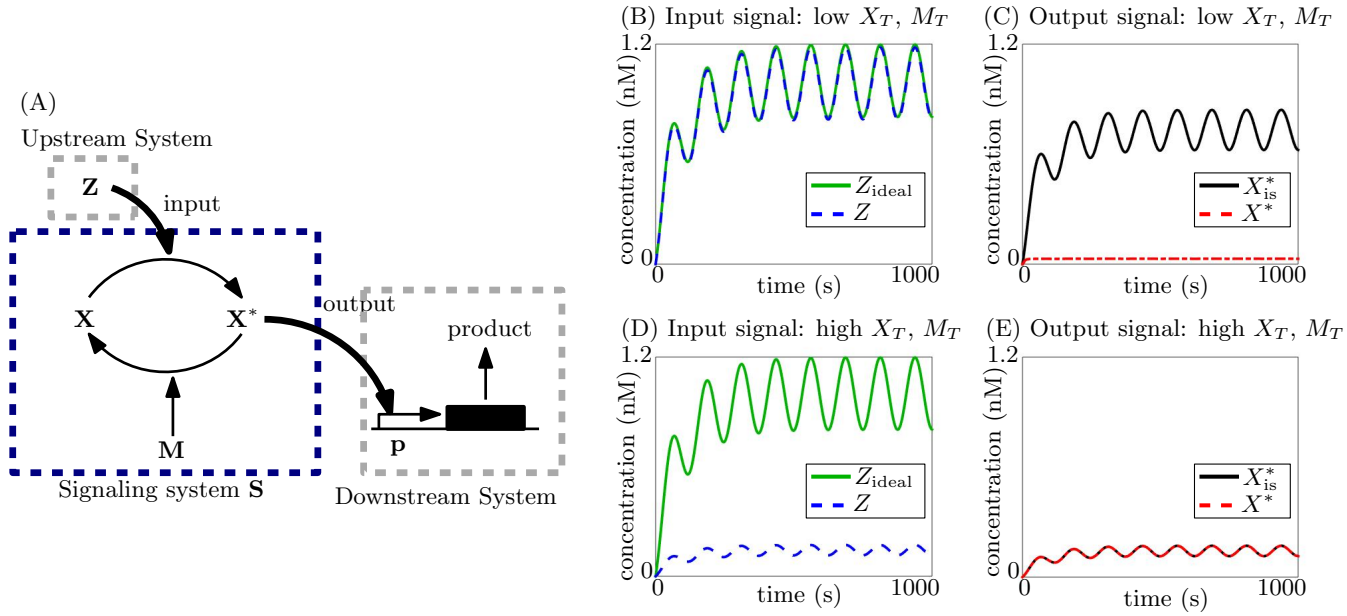


Fig 2. Tradeoff between small retroactivity to the input and attenuation of retroactivity to the output in a single phosphorylation cycle. (A) Single phosphorylation cycle, with input Z as the kinase: X is phosphorylated by Z to X^*, and dephosphorylated by the phosphatase M. X^* is the output and acts on sites p in the downstream system, which is depicted as a gene expression system here. (B)-(E) Simulation results for ODE model shown in SI Section 5.2 eqn. (19). Common simulation parameters [1]: $k(t) = 0.01(1 + sin(0.05t))$, $\delta = 0.01s^{-1}$, $k_1 = k_2 = 600s^{-1}$, $a_1 = a_2 = 18nM^{-1}s^{-1}$, $d_1 = d_2 = 2400s^{-1}$, $k_{\text{on}} = 10nM^{-1}s^{-1}, k_{\text{off}} = 10s^{-1}$. (B) Effect of retroactivity to the input with low substrate concentration X_T: for ideal input Z_{ideal}, system is simulated with $X_T = M_T = p_T = 0$; for actual input Z, system is simulated with $X_T = M_T = 10nM$, $p_T = 100nM$. (C) Effect of retroactivity to the output with low substrate concentration X_T: for isolated output X^*_{is}, system is simulated with $X_T = M_T = 10nM$, $p_T = 0$; for actual output X^*, system is simulated with $X_T = M_T = 10nM$, $p_T = 100nM$. (D) Effect of retroactivity to the input with high substrate concentration X_T: for ideal input Z_{ideal}, system is simulated with $X_T = M_T = p_T = 0$; for actual input Z, system is simulated with $X_T = M_T = 1000nM$, $p_T = 100nM$. (E) Effect of retroactivity to the output with high substrate concentration X_T: for isolated output X^*_{is}, system is simulated with $X_T = M_T = 1000nM$, $p_T = 0$; for actual output X^*, system is simulated with $X_T = M_T = 1000nM$, $p_T = 100nM$.

Since X_T is low, this sequestration results in a large relative change in the amount of total substrate available for the cycle, and thus interconnection to the downstream system has a large effect on the behavior of the cycle. For the case when X_T (and M_T) is high, the system shows exactly the opposite behavior. From Fig. 2D, we see that $\mathcal{R}$ is high (thus not satisfying requirement (i) of Def. 1), since the kinase must phosphorylate a large amount of substrate, but $\mathcal{S}$ is attenuated (satisfying requirement (ii)) since there is enough total substrate available for the cycle even once X^* is sequestered. Thus, this system shows a trade-off: by increasing X_T (and M_T) we attenuate retroactivity to the output but to the cost of increasing retroactivity to the input. Similarly, by decreasing X_T (and M_T), we make retroactivity to the input smaller, but to the cost of being unable to attenuate retroactivity to the output. Therefore, requirements (i) and (ii) cannot be independently obtained by tuning X_T and M_T.

We note that because the signaling reactions, i.e., phosphorylation and dephosphorylation, act on a faster timescale than the input, the signaling system operates at quasi-steady state and the output is able to quickly catch up to changes in the input. It has been demonstrated in [32], [34] that this fast timescale of operation of the signaling system attenuates the temporal effects of retroactivity to the output, which would otherwise result in the output slowing down in the presence of the downstream system. Thus, while the high substrate concentration X_T is required to reduce the effect of retroactivity to the output due to permanent sequestration, timescale separation is necessary for attenuating the temporal effects of the binding-unbinding reaction flux [32].

[1] Association, dissociation and catalytic rate constants (a_i, d_i, k_i) and range of total protein concentrations taken from [35]

2.1 General mathematical model and main theorems

The single phosphorylation cycle, while showing some ability to attenuate retroactivity, is not able to transmit unidirectional signals due to the trade-off seen above. We therefore study, with respect to unidirectional signal transmission, different architectures of signaling systems, composed of phosphorylation cycles and phosphotransfer systems which are ubiquitous in natural signal transduction [1]- [7], [14]- [19]. To this end, we first layout the following general ODE model, using reaction-rate equations, that describes any signaling system architecture in the interconnection topology of Fig. 1A:

$$\begin{aligned}
\frac{dU}{dt} &= f_0(U, R\underline{X}, S_1 v, t) + G_1 A \underline{r}(U, \underline{X}, S_2 v),\\
\frac{d\underline{X}}{dt} &= G_1 B \underline{r}(U, \underline{X}, S_2 v) + G_1 f_1(U, \underline{X}, S_3 v) + G_2 C s(\underline{X}, v),\\
\frac{dv}{dt} &= G_2 D s(\underline{X}, v),\\
Y &= I\underline{X}.
\end{aligned} \tag{1}$$

Here, the variable t represents time, U is the input signal (the concentration of the input species), $\underline{X}$ is a vector of concentrations of the species of the signaling system, Y is the output signal (the concentration of the output species) and v is the state variable of the downstream system. In the cases that follow, v is the normalized concentration of the complex formed by the output species Y and its target binding sites p in the downstream system. The positive scalar G_1 captures the timescale separation between the reactions of the signaling system and the dynamics of the input. Since we consider relatively slow inputs, we have that $G_1 \gg 1$. The positive scalar G_2 captures the timescale separation between the binding-unbinding rates between the output Y and its target sites p in the downstream system and the dynamics of the input. Since binding-unbinding reactions also operate on a fast timescale, we have that $G_2 \gg 1$. We define $\epsilon = \max\left(\frac{1}{G_1}, \frac{1}{G_2}\right)$ and thus, $\epsilon \ll 1$. Further, the matrices A, B, C and D are constant stoichiometric matrices [42], and f_0 and f_1 are reaction-rate vectors. The SI Section 5.1 contains a formal treatment of this multi-timescale system.

The retroactivity to the input $\mathcal{R}$ indicated in Fig. 1A equals $(R, \underline{r}, S_1)$. Here, the parameter R accounts for decay/degradation of complexes formed by the input species with species of the signaling system, thus leading to an additional channel for removal of the input species through their interaction with the signaling system. Similarly, scalar S_1 represents decay of complexes formed by the input species with species of the downstream system. This additional decay leads to an effective increase in decay of the input, thus affecting its steady-state. The reaction-rate vector $\underline{r}$ is the reaction flux resulting from the reactions between species of the upstream system and those of the signaling system. This additional reaction flux affects the temporal behavior of the input, often slowing it down, as demonstrated previously [11]. The retroactivity to the output $\mathcal{S}$ of Fig. 1A equals (S_1, S_2, S_3, s). As species of the signaling system are sequestered by the downstream system, their free concentration changes. This is accounted for by the vectors S_2 and S_3. The reaction rate vector s represents the additional reaction flux due to the binding-unbinding of the output protein with the target sites in the downstream system. For ideal unidirectional signal transmission, the effects of $\mathcal{R}$ and $\mathcal{S}$ must be small. The ideal input of Fig. 1B, U_{ideal}, is the input when retroactivity to the input $\mathcal{R}$ is zero, i.e., when $R = S_1 = \underline{r} = 0$. The isolated output of Fig. 1C, Y_{is}, is the output when retroactivity to the output $\mathcal{S}$ is zero, i.e., when $S_1 = S_2 = S_3 = s = 0$.

In order to provide the main theoretical result of this paper, which provides conditions for which system (1) satisfies Def. 1, it is useful to introduce some definitions. We let $v = \phi(\underline{X})$ denote the solution to $s(\underline{X}, v) = 0$. Since $G_2 \gg 1$, this captures the quasi-steady state concentration of v. Similarly, we let $\underline{X} = \underline{\Psi}(U, v)$ denote the solution to $Br(U, \underline{X}, S_2 v) + f_1(U, \underline{X}, S_3 v) = 0$. Since $G_1 \gg 1$, this captures the quasi-steady state concentration of the species of the signaling system $\underline{X}$. Finally, we let $\underline{X} = \underline{\Gamma}(U)$ denote the solution to $B\underline{r}(U, \underline{X}, S_2\phi(\underline{X})) + f_1(U, \underline{X}, S_3\phi(\underline{X})) = 0$. For the isolated system as shown in Fig. 1C, we let $\underline{X} = \underline{\Gamma}_{\text{is}}(U_{\text{is}})$ denote the solution to $B\underline{r}(U_{\text{is}}, \underline{X}, 0) + f_1(U_{is}, \underline{X}, 0) = 0$. Further, it can be shown that there exists a function $g(S_2, S_3)$, such that $g(S_2, S_3)$ decreases as $|S_2|$ and $|S_3|$ decrease, and is zero when $S_2 = S_3 = 0$ (details in SI Section 5.1). This function captures the dependence of the difference $|\underline{\Gamma}(U) - \underline{\Gamma}_{\text{is}}(U)|$ on S_2 and S_3. We further assume that there exist invertible matrices T and Q, and matrices M and P such that $TA + MB = 0$, $Mf_1 = 0$ and $QC + PD = 0$. The assumptions and lemmas that use singular perturbation and contraction theory to arrive at the results that follow are given in SI Section 5.1. For system (1), for some fixed positive constants L_0, L_Ψ, L_Γ (definitions in SI Section 5.1), we then have the following results.

The first theorem provides an upper-bound on the effect of the retroactivity to the input for system (1).

Theorem 1. *The effect of retroactivity to the input is given by:*

$$|U_{ideal}(t) - U(t)| \leq \frac{h_1 + h_2 + h_3}{\lambda} + \mathcal{O}(\epsilon), \quad \textit{for } t \in [t_b, t_f],$$

where $h_1 = \sup_U \ L_0|R\underline{\Gamma}(U)|, \qquad h_2 = \sup_U \ L_0|S_1\phi(\underline{\Gamma}(U))|,$

$$h_3 = \sup_{U, t\in[t_b,t_f]} \left| \underbrace{\left(T^{-1}M\frac{\partial \underline{\Gamma}(U)}{\partial U} + T^{-1}MQ^{-1}P\frac{\partial \phi}{\partial \underline{X}}\Big|_{\underline{X}=\underline{\Gamma}(U)} \frac{\partial \underline{\Gamma}(U)}{\partial U} \right)}_{a} \frac{dU}{dt} \right|.$$

The next theorem provides an upper-bound on the effect of retroactivity to the output for system (1).

Theorem 2. *The effect of retroactivity to the output is given by:*

$$|Y_{is}(t) - Y(t)| \leq ||I||\bar{h}_1 + ||I||L_\Gamma \frac{h_2 + \bar{h}_3}{\lambda} + \mathcal{O}(\epsilon), \quad \textit{for } t \in [t_f, t_b],$$

where $\bar{h}_1 = \sup_U \ L_\Psi\, |g(S_2, S_3)\phi(\underline{\Gamma}(U))|, \qquad h_2 = \sup_U \ L_0|S_1\phi(\underline{\Gamma}(U))|,$

$$\bar{h}_3 = \sup_{U, t\in[t_b,t_f]} \left| \underbrace{\left(T^{-1}MQ^{-1}P\frac{\partial \phi(\underline{X})}{\partial \underline{X}}\Big|_{\underline{X}=\underline{\Gamma}(U)} \frac{\partial \underline{\Gamma}(U)}{\partial U} \right)}_{b} \frac{dU}{dt} \right|.$$

The final theorem gives an expression for the input-output relationship of system (1).

Theorem 3. *The relationship between* $Y_{is}(t)$ *and* $U_{is}(t)$ *is given by:*

$$Y_{is}(t) = I\underline{\Gamma}_{is}(U_{is}(t)) + \mathcal{O}(\epsilon), \textit{ for } t \in [t_b, t_f].$$

Theorem 1 provides an upper-bound on $|U_{\text{ideal}}(t) - U(t)|$ in terms of expressions h_1, h_2 and h_3. These terms can be made small making $|R\underline{\Gamma}|$, S_1 and a small. We will seek to make these terms small by tuning the total protein concentrations. For example, for the single phosphorylation cycle of Fig. 2A where the input U equals Z,

$$|R\underline{\Gamma(U)}| = \frac{X_T}{K_{m1}}Z, S_1 = 0 \text{ and } a = \frac{X_T}{K_{m1}},$$

when $K_{m1}, K_{m2} \gg Z$; where K_{m1} is the Michaelis-Menten constant of the phosphorylation reaction and K_{m2} is the Michaelis-Menten constant of the dephosphorylation reaction (details in result (i) of SI Section 5.2). Thus, using Theorem 1, we find that as X_T is made small, $|U_{\text{ideal}}(t) - U(t)|$ is made small, thus satisfying requirement (i) of Def. 1.

Similarly, Theorem 2 provides an upper-bound on $|Y_{\text{is}}(t) - Y(t)|$ in terms of $\bar{h}_1, h_2, \bar{h}_3$, which can be made small by making S_1, S_2, S_3 and b small. For the single phosphorylation cycle, where output Y equals X^*, we find that (details in result (ii) of SI Section 5.2)

$$S_1 = 0, S_2 = \frac{p_T}{X_T}, S_3 = \frac{\delta p_T}{a_2 M_T} \text{ and } b = 0,$$

where δ is the rate of dilution and a_2 is the rate of association of X* and M. Thus, using Theorem 2, we find that as X_T and M_T are made large, $|Y_{\text{is}}(t) - Y(t)|$ is made small, thus satisfying requirement (ii) of Def. 1. Finally, condition (iii) of Definition 1 can be analyzed using Theorem 3, which provides an expression for the output, $I\underline{\Gamma}_{\text{is}}(U_{\text{is}})$. For the single phosphorylation cycle, this evaluates to (from eqn. (23) in SI Section 5.2):

$$X^*_{\text{is}}(t) \approx \underline{\Gamma}(Z_{\text{is}}(t)) \approx \frac{k_1 K_{m2}}{k_2 K_{m1}} \frac{X_T}{M_T} Z_{\text{is}}(t),$$

when $K_{m1}, K_{m2} \gg Z$. Using this expression, M_T can be tuned in proportion to X_T to satisfy requirement (iii) of Def. 1 with $m = 1$ for some desired input-output gain K.

This way, the above theorems can be used to identify ways to tune the total protein concentration of a signaling system such that it satisfies Def. 1. Thus, based on Theorems 1, 2 and 3, we analyze the following signaling architectures: a double phosphorylation cycle with kinase as input, a phosphotransfer system where the phosphate donor is phosphorylated by the input kinase, a cascade of single phosphorylation cycles, a phosphotransfer system where the input is the phosphate donor that undergoes autophosphorylation, a single phosphorylation cycle with a substrate as input, and a double phosphorylation cycle with a substrate as input.

2.2 Double phosphorylation cycle with input as kinase

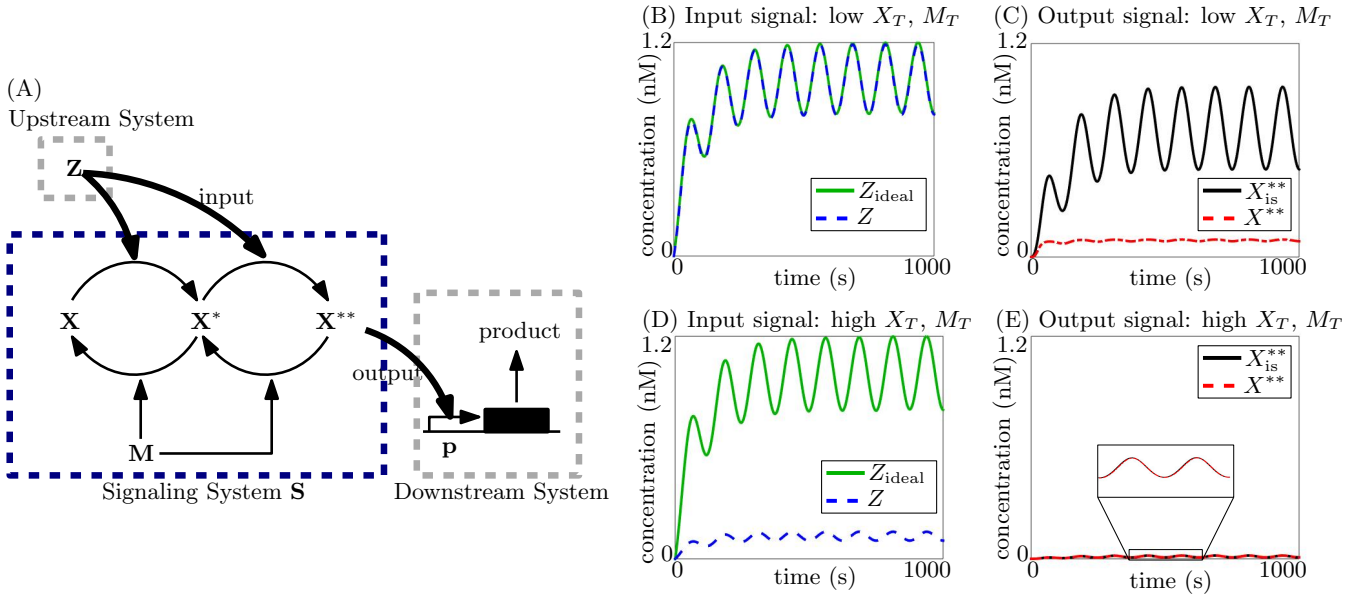


Fig 3. Tradeoff between small retroactivity to the input and attenuation of retroactivity to the output in a double phosphorylation cycle. (A) Double phosphorylation cycle, with input Z as the kinase: X is phosphorylated by Z to X^*, and further on to X^{**}. Both these are dephosphorylated by the phosphatase M. X^{**} is the output and acts on sites p in the downstream system, which is depicted as a gene expression system here. (B)-(E) Simulation results for ODE model (31) shown in SI Section 5.3. Common simulation parameters [1]: $k(t) = 0.1(1 + sin(0.05t)), \delta = 0.01s^{-1}$, $k_1 = k_2 = k_3 = k_4 = 600s^{-1}, a_1 = a_2 = a_3 = a_4 = 18nM^{-1}s^{-1}, d_1 = d_2 = d_3 = d_4 = 2400s^{-1}, k_{\text{on}} = 10nM^{-1}s^{-1}, k_{\text{off}} = 10s^{-1}$. (B) Effect of retroactivity to the input with low substrate concentration X_T: ideal input Z_{ideal} is simulated with $X_T = M_T = p_T = 0$, actual input Z is simulated with $X_T = 100nM$, $M_T = 10nM$, $p_T = 100nM$. (C) Effect of retroactivity to the output with low substrate concentration X_T: for isolated output X^{**}_{is}, system is simulated with $X_T = 10nM$, $M_T = 3nM$, $p_T = 0$, for actual output X^{**}, system is simulated with $X_T = 10nM$, $M_T = 3nM$, $p_T = 100nM$. (D) Effect of retroactivity to the input with high substrate concentration X_T: for ideal input Z_{ideal}, system is simulated with $X_T = M_T = p_T = 0$, for actual input Z, system is simulated with $X_T = 1200nM, M_T = 39nM, p_T = 100nM$. (E) Effect of retroactivity to the output with high substrate concentration X_T: for isolated output X^{**}_{is}, system is simulated with $X_T = 1200nM, M_T = 39nM, p_T = 0$, for actual output X^{**}, system is simulated with $X_T = 1200nM, M_T = 39nM, p_T = 100nM$.

Here, we consider a double phosphorylation cycle with a common kinase Z for both phosphorylation cycles as the input and the doubly phosphorylated substrate X^{**} as the output. This architecture is found in the second and third stages of the MAPK cascade, where the kinase phosphorylates both the threonine and tyrosine sites in a distributive process [43]. This configuration is shown in Fig. 3A. Referring to Fig. 1A, the input signal U is the concentration Z of the kinase and the output signal Y is the concentration X^{**} of the doubly phosphorylated substrate X.

The input kinase is produced at a time-varying rate $k(t)$. All species dilute with a rate constant δ, and the total promoter concentration in the downstream system is p_T. The total substrate and phosphatase concentrations are X_T and M_T, respectively. The Michaelis-Menten constants for the two phosphorylation and the two dephosphorylation reactions are K_{m1}, K_{m3}, K_{m2} and K_{m4}, respectively. The catalytic reaction rate constants of these reactions are k_1, k_3, k_2 and k_4, respectively. The system's chemical reactions are shown in SI Section 5.3 eqns. (30). As explained before, the parameters

that we tune to investigate retroactivity effects are the total protein concentrations of the phosphorylation cycle, that is, X_T and M_T. Specifically, using Theorems 1, 2 and 3, we tune X_T and M_T to verify if this system can transmit a unidirectional signal, according to Definition 1. We therefore find what follows.

(i) Retroactivity to the input: In Theorem 1, we provided an upper bound, $\frac{h_1+h_2+h_3}{\lambda}$, on $|U_{\text{ideal}}(t) - U(t)|$, which is the term that must be small to satisfy requirement (i) of Def. 1, i.e., to have a small retroactivity to the input. For this system, λ does not depend on X_T and M_T. Further, we find that $h_2 = 0$, and that to make h_1 and h_3 small, we must have small $\frac{X_T}{K_{m1}}$ and small $\frac{X_T}{M_T K_{m3}} \frac{k_1 K_{m2}}{k_2 K_{m1}}$. Thus, to have small retroactivity to the input, the parameter X_T must be small. (Mathematical details to derive these expressions are in result (i) of SI Section 5.3).

(ii) Retroactivity to the output: In Theorem 2, we provided an upper bound on $|Y_{\text{is}}(t) - Y(t)|$. To satisfy requirement (ii) of Def. 1, i.e., to attenuate retroactivity to the output, this upper bound, $\frac{\bar{h}_1+h_2+\bar{h}_3}{\lambda}$, must be made small. For this system, we find that $h_2 = 0$ and $\bar{h}_3 = 0$. Further, to make $\bar{h}_1$ small, we must have a small $\frac{p_T}{X_T}$. Thus, to attenuate retroactivity to the output, we must have a large X_T. (Mathematical details to derive these expressions are in result (ii) of SI Section 5.3).

(iii) Input-output relationship: In Theorem 3, we found an approximate expression for the input-output relationship, i.e., $Y_{\text{is}} \approx I\underline{\Gamma}_{\text{is}}(U_{\text{is}})$. We use this to find that the $X_{\text{is}}^{**} \approx \frac{k_1 k_3 K_{m2} K_{m4}}{k_2 k_4 K_{m1} K_{m3}} \frac{X_T}{M_T^2} Z_{\text{is}}^2$, when $K_{m1}, K_{m2}, K_{m3}, K_{m4} \gg Z_{\text{is}}$, $K_{m2} \gg X_{\text{is}}^*$, $K_{m4} \gg X_{\text{is}}^{**}$ and $M_T \gg Z_{\text{is}}$. Under these assumptions, this system satisfies requirement (iii) of Def. 1 by tuning the ratio $\frac{X_T}{M_T^2}$ to achieve a desired K with $m = 2$. (Mathematical details to derive these expressions are in result (iii) of SI Section 5.3, eqn. (41)).

This system shows a similar trade-off between properties (i) and (ii) as the single phosphorylation cycle. Retroactivity to the input is large when substrate concentration X_T (and M_T) increases, because the input Z must phosphorylate a large amount of substrate thus leading to a large reaction flux to Z due to the phosphorylation reaction. However, if X_T (and M_T) is made small, the system cannot attenuate the retroactivity to the input, since as the output X^{**} is sequestered by the downstream system, there is not enough substrate available for the signaling system. Therefore, requirements (i) and (ii) cannot be independently satisfied.

These mathematical predictions can be appreciated from the numerical simulations of Figs. 3B-3E and this result is summarized in Fig. 9B.

2.3 Phosphotransfer with phosphate donor phosphorylated by the input kinase

We now consider a signaling system composed of a phosphotransfer system, whose phosphate donor receives the phosphate group via phosphorylation through a kinase Z. Instances of phosphotransfer systems include the reaction between YPD1 and SKN7 [44], which is a central component of the osmotic stress response of yeast. Such a system was also implemented as a synthetic insulation device in [34], where kinase JH1 phosphorylates STAT5-HKRR, which then transfers the phosphate group to YPD1 through phosphotransfer. This architecture is shown in Fig. 4A. In this case, the input signal U of Fig. 1A is Z, which is the concentration of kinase Z that phosphorylates the phosphate donor X_1, which then transfers the phosphate group to protein X_2. The output signal Y in Fig. 1A is then X_2^*, which is the concentration of the phosphorylated substrate X_2^*. Protein X_2^* is dephosphorylated by phosphatase M. Total concentrations of proteins X_1, X_2 and M are X_{T1}, X_{T2} and M_T, respectively. The Michaelis-Menten constants for the phosphorylation of X_1 by Z and dephosphorylation of X_2^* by M are K_{m1} and K_{m3}, and the catalytic rate constants of these are k_1 and k_3, respectively. The association rate constant of complex formation by X_2^* and X_1 is a_3. These reactions are shown in eqns. (46) in SI Section 5.4. The total concentration of promoter sites in the downstream system is p_T. The input Z is produced at a time-varying rate $k(t)$. As before, the parameters we change to analyze the system for unidirectional signal transmission are its total protein concentrations, X_{T1}, X_{T2} and M_T. Using Theorems 1, 2 and 3, we analyze the system's ability to transmit unidirectional signals as per Definition 1 as X_{T1}, X_{T2} and M_T are varied. This is done as follows.

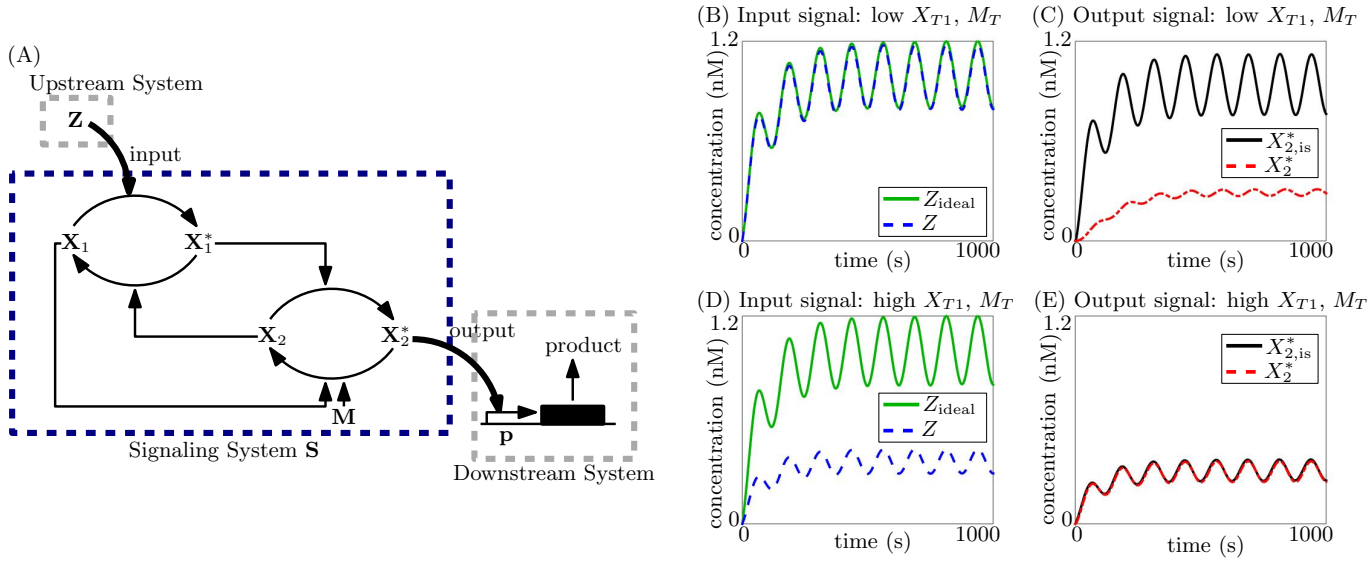


Fig 4. Tradeoff between small retroactivity to the input and attenuation of retroactivity to the output in a phosphotransfer system. (A) System with phosphorylation followed by phosphotransfer, with input Z as the kinase: Z phosphorylates X_1 to X_1^*. The phosphate group is transferred from X_1^* to X_2 by a phosphotransfer reaction, forming X_2^*, which is in turn dephosphorylated by the phosphatase M. X_2^* is the output and acts on sites p in the downstream system, which is depicted as a gene expression system here. (B)-(E) Simulation results for ODE (47) in SI Section 5.4. Common parameters[1]: $k(t) = 0.01(1 + sin(0.05t))$, $\delta = 0.01s^{-1}, k_1 = k_2 = k_4 = 15s^{-1}, a_1 = a_2 = a_3 = a_4 = 18nM^{-1}s^{-1}$, $d_1 = d_2 = d_3 = d_4 = 2400s^{-1}, k_{\text{on}} = 10nM^{-1}s^{-1}, k_{\text{off}} = 10s^{-1}$. (B) Effect of retroactivity to the input with low substrate concentration X_{T1}: for ideal input Z_{ideal}, system is simulated with $X_{T1} = X_{T2} = M_T = p_T = 0$; for actual input Z, system is simulated with $X_{T1} = M_T = 3nM$, $X_{T2} = 1200nM$, $p_T = 100nM$. (C) Effect of retroactivity to the output with low substrate concentration X_{T1}: for isolated output $X^*_{2,\text{is}}$, system is simulated with $X_{T1} = M_T = 3nM$, $X_{T2} = 1200nM, p_T = 0$; for actual output X_2^*, system is simulated with $X_{T1} = M_T = 3nM$, $X_{T2} = 1200nM$, $p_T = 100nM$. (D) Effect of retroactivity to the input with high substrate concentration X_{T1}: for ideal input Z_{ideal}, system is simulated with $X_{T1} = X_{T2} = M_T = p_T = 0$; for actual input Z, system is simulated with $X_{T1} = M_T = 300nM$, $X_{T2} = 1200nM$, $p_T = 100nM$. (E) Effect of retroactivity to the output with high substrate concentration X_{T1}: for isolated output $X^*_{2,\text{is}}$, system is simulated with $X_{T1} = M_T = 300nM$, $X_{T2} = 1200nM$, $p_T = 0$; for actual output X_2^*, system is simulated with $X_{T1} = M_T = 300nM$, $X_{T2} = 1200nM$, $p_T = 100nM$.

(i) Retroactivity to the input: As before, we minimize the terms h_1, h_2 and h_3 as described in Theorem 1 to have a small retroactivity to the input and satisfy requirement (i) of Def. 1. We find that $h_2 = 0$ and that for small h_1 and h_3, we must have small $\frac{X_{T1}}{K_{m1}}$. Thus, for small retroactivity to the input, we must have small X_{T1}. (Mathematical details to derive these expressions are in result (i) of SI Section 5.4).

(ii) Retroactivity to the output: To satisfy requirement (ii) of Def. 1, i.e., to attenuate retroactivity to the output, we must have small $\bar{h}_1$, h_2 and $\bar{h}_3$ as defined in Theorem 2. We find that for this system $h_2 = 0$ and $\bar{h}_3 = 0$. Further, for $\bar{h}_1$ to be small, $\frac{p_T}{X_{T2}}$ and $\frac{\delta p_T}{a_3 X_{T1}}$ must be small. Thus, for a small retroactivity to the output, we must have large X_{T1} and X_{T2}. (Mathematical details to derive these expressions are in result (ii) of SI Section 5.4).

(iii) Input-output relationship: Using the expression for the input-output relationship given by Theorem 3, we find that $X_2^* \approx \frac{k_1 K_{m3}}{k_3 K_{m1}} \frac{X_{T1}}{M_T} Z$ when $K_{m1} \gg Z_{\text{is}}$ and $K_{m4} \gg X^*_{2,\text{is}}$. Under these assumptions, this system satisfies requirement (iii) of Def. 1 by tuning the ration $\frac{X_{T1}}{M_T}$ with $m = 1$. (Mathematical details to derive these expressions are in result (iii) of SI Section 5.4, eqn. (51)).

In light of (i) and (ii), we note that the system shows a trade-off in attenuating retroactivity to the input and output. Retroactivity to the input can be made small, by making X_{T1} (and M_T) small, since kinase Z must phosphorylate less substrate. However, the system with low X_{T1} is unable to attenuate retroactivity to the output, which requires that X_{T1} be large. This is because, as the output X_2^* is sequestered by the downstream system and undergoes decay as a complex, this acts as an additional channel of removal for the phosphate group from the system, which was received from X_1^*. If X_{T1} (and M_T) is small, this removal of the phosphate group affects the amount of X_1^* in the system to a larger extent that when X_{T1} is large. Thus, there exists a trade-off between requirements (i) and (ii) of Def. 1. Further, in these two

cases (large X_{T1} and small X_{T1}), we vary M_T in proportion to X_{T1} to satisfy requirement (iii) of Def. 1.

This mathematical analysis is demonstrated in the simulation results shown in Figs. 4B-4E and the discussion is summarized in Fig. 9B.

2.4 Cascade of single phosphorylation cycles

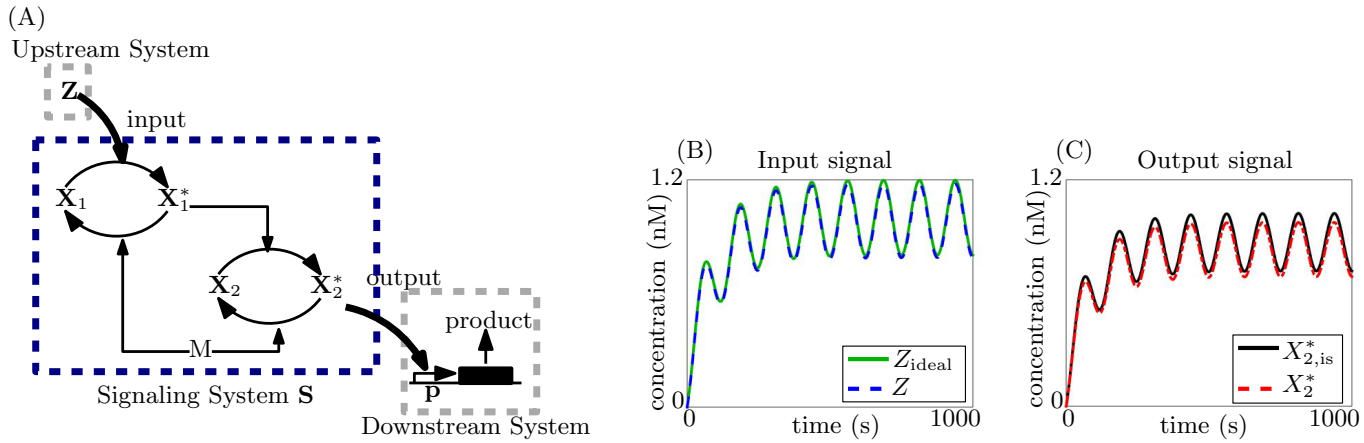


Fig 5. Tradeoff between small retroactivity to the input and attenuation of retroactivity to the output is overcome by a cascade of single phosphorylation cycles. (A) Cascade of 2 phosphorylation cycles that with kinase Z as the input: Z phosphorylates X_1 to X_1^*, X_1^* acts as the kinase for X_2, phosphorylating it to X_2^*, which is the output, acting on sites p in the downstream system, which is depicted as a gene expression system here. Both X_1^* and X_2^* are phosphorylated by phosphatase M. (B), (C) Simulation results for ODEs (61)-(78) in SI Section 5.5 with N = 2. Simulation parameters[1]: $k(t) = 0.01(1 + sin(0.05t))nM.s^{-1}, \delta = 0.01s^{-1}, a_1 = a_2 = 18(nM.s)^{-1}, d_1 = d_2 = 2400s^{-1}, k_1 = k_2 = 600s^{-1}$. (B) Effect of retroactivity to the input: for the ideal input Z_{ideal}, system is simulated with $X_{T1} = X_{T2} = M_T = p_T = 0$; for actual input Z, system is simulated with $X_{T1} = 3nM$, $X_{T2} = 1000nM$, $M_T = 54nM$, $p_T = 100nM$. (C) Effect of retroactivity to the output: for the isolated output Y_{is}, system is simulated with $X_{T1} = 3nM$, $X_{T2} = 1000nM$, $M_T = 54nM$, $p_T = 0$; for the actual output, system is simulated with $X_{T1} = 3nM$, $X_{T2} = 1000nM$, $M_T = 54nM, p_T = 100nM$.

We have now seen three systems that show a trade-off between attenuating retroactivity to the output and imparting a small retroactivity to the input: the single phosphorylation cycle, the double phosphorylation cycle and the phosphotransfer system, all with a kinase as input. In all three cases, the trade-off is due to the fact that, as the total substrate concentration is increased to attenuate the effect of retroactivity on the output, the system applies a large retroactivity to the input. Thus, the requirements (i) and (ii) of Def. 1 cannot be independently achieved. In [34], a cascade of phosphotransfer systems was found to apply a small retroactivity to the input and to attenuate retroactivity to the output. Further, cascades of single and double PD cycles are ubiquitous in cellular signaling, such as in the MAPK cascade [14], [45]. Motivated by this, here we consider a cascade of PD cycles to determine how a cascaded architecture can overcome this trade-off. We have found that single and double PD cycles, and the phosphotransfer system, show similar properties with respect to unidirectional signal transmission. Thus, our findings are applicable to all systems composed of cascades of single stage systems, such as the single PD cycle, the double PD cycle and the phosphotransfer system analyzed in Section 2.3 (simulation results for cascades of different systems are in SI 5.5 Fig. 11 and Fig. 12).

We consider a cascade of two single phosphorylation cycles, shown in Fig. 5A. The input signal is Z, the concentration of kinase Z. Z phosphorylates substrate X_1 to X_1^*, which acts as a kinase for substrate X_2, phosphorylating it to X_2^*. Both X_1^* and X_2^* are dephosphorylated by a common phosphatase M. The output signal is X_2^*, the concentration of X_2^*.

The input Z is produced at a time-varying rate $k(t)$, and all species dilute with rate constant δ. The substrate of the cycles are produced at constant rates k_{X1} and k_{X2}, respectively, and the phosphatase is produced at a constant rate k_M. We then define $X_{T1} = \frac{k_{X1}}{\delta}$, $X_{T2} = \frac{k_{X2}}{\delta}$ and $M_T = \frac{k_M}{\delta}$. The concentration of promoter sites in the downstream system is p_T. The Michaelis-Menten constants for the phosphorylation and dephosphorylation reactions are K_{m1} and K_{m2}, respectively (assuming identical reaction-rate parameters for both cycles), and catalytic rate constants are k_1 and k_2. The chemical reactions for this system are shown in eqns. (54)-(60) in SI Section 5.5. As before, the parameters we vary to

analyze this system's ability to transmit unidirectional signals are X_{T1}, X_{T2} and M_T. Using Theorems 1, 2 and 3, we seek to tune these to satisfy the requirements of Def. 1. We find what follows.

(i) Retroactivity to the input: To satisfy requirement (i) of Def. 1, we must have small h_1, h_2 and h_3 as defined in Theorem 1. For this system, we find that $h_1 = h_2 = 0$. We further find that to make d_3 small, $\frac{X_{T1}}{K_{m1}}$ must be small. Thus, to have a small retroactivity to the input, X_{T1} must be small. (Mathematical details to derive these expressions are in result (i) of SI Section 5.5).

(ii) Retroactivity to the output: As before, we minimize $\bar{h}_1$, h_2 and $\bar{h}_3$ from Theorem 2 to satisfy requirement (ii) of Def. 1, i.e., attenuating retroactivity to the output. We find that $h_2 = 0$ and $\bar{h}_3 = 0$. Further, to make $\bar{h}_1$, we must have a small $\frac{p_T}{X_{T2}}$. Thus, to attenuate retroactivity to the output, X_{T2} must be large. (Mathematical details to derive these expressions are in result (ii) of SI Section 5.5).

(iii) Input-output relationship: Using the expression found in Theorem 3, we find that the input-output relationship is $X^*_{2,\text{is}} \approx (\frac{k_1 K_{m2}}{k_2 K_{m1}})^2 \frac{X_{T1} X_{T2}}{M_T^2} Z_{\text{is}}$ when $K_{m1}, K_{m2} \gg Z_{\text{is}}$. The ratio $\frac{X_{T1} X_{T2}}{M_T^2}$ can thus be tuned such that the system satisfies (iii) of Def. 1 with $m = 1$. However, as $\frac{X_{T2}}{X_{T1}}$ increases beyond a point, the second stage of the cascade affects the first stage, and the output begins to saturate with respect to the input, thus not satisfying requirement (iii). In SI 5.5, we have shown that this non-linearity can be reduced by additional cycles, between the first and second cycle, in the cascade up to a certain number of cycles. That is, there exists an optimal number of cycles in the cascade for which the term leading to a non-linear input-output response (shown in eqn. (82) in SI Section 5.5) is minimized. This is because, each downstream cycle affects the response of the cycle directly upstream to it, making it non-linear. For each cycle, these non-linearities add up, and thus the number of terms contributing to the total non-linearity increase with the number of cycles. However, additional cycles reduce the non-linear effect of each individual stage. These two opposing effects make it so that the net non-linearity in the output of the final stage has an optimum. (Mathematical details to derive these expressions are in result (iii) of SI Section 5.5, eqn. (81)).

We thus note that the trade-off between attenuating retroactivity to the output and imparting small retroactivity to the input, found in single-stage systems is broken by having a cascade of two cycles. This is because the input kinase Z only directly interacts with the first cycle, and thus when X_{T1} is made small, the upstream system faces a small reaction flux due to the phosphorylation reaction, making retroactivity to the input small. The downstream system sequesters the species X_2^*, and when X_{T2} is made high, there is enough substrate X_2 available for the signaling system to be nearly unaffected, thus attenuating retroactivity to the output. This is verified in Figs. 5B,5C. The trade-off found in the single cycle in Figs. 2B-2E is overcome by the cascade, where we have tuned M_T to satisfy requirement (iii) of Def. 1. When the total substrate concentration for a single cycle is low, the retroactivity to the input is small (Fig. 2B) but the retroactivity to the output is not attenuated (Fig. 2C). When the total substrate concentration of this cycle is increased, the retroactivity to the output is attenuated (Fig. 2D) but the input, and therefore the output, are highly changed due to an increase in the retroactivity to the input (Figs. 2D, 2E). When the same two cycles are cascaded, with the low substrate concentration cycle being the first and the high substrate concentration cycle being the second (and M_T tuned to maintain the same gain K as the single cycles), retroactivity to the input is small and retroactivity to the output is attenuated (Figs. 5B, 5C). Thus, cascading two cycles overcomes the trade-off found in a single cycle.

These results are summarized in Fig. 9E. While the system demonstrated here is a cascade of single phosphorylation cycles, the same decoupling is true for cascaded systems composed of double phosphorylation cycles and phosphorylation cycles followed by phosphotransfer, which as we saw in the previous subsections, show a similar kind of trade-off. Cascades of such systems, with the first system with a low substrate concentration and the last system with a high substrate concentration thus both, impart a small retroactivity to the input, and attenuate retroactivity to the output and are therefore able to transmit unidirectional signals. This can be seen via simulation results in SI Section 5.5, where a cascade of a phosphotransfer system and a single PD cycle is seen in Fig. 11 and a cascade of a single PD cycle and a double PD cycle is seen in Fig. 12.

2.5 Phosphotransfer with the phosphate donor undergoing autophosphorylation as input

Here, we consider a signaling system composed of a protein X_1 that undergoes autophosphorylation and then transfers the phosphate group to a substrate X_2, shown in Fig. 6A. An instance of this system is found in the bacterial chemotaxis system, where the protein CheY acquires a phosphate group through a phosphotransfer reaction with CheA, which is a histidine kinase that first undergoes autophosphorylation [46]. The input signal U of Fig. 1A is X_1, the concentration of

protein X_1 which undergoes autophosphorylation, and the output signal Y of Fig. 1A is X_2^*, the concentration of phosphorylated protein X_2^*. The total protein concentrations of substrate X_2 and phosphatase M are X_{T2} and M_T, respectively. The total concentration of promoters in the downstream system is p_T. Autophosphorylation of a protein typically follows a conformational change that either allows the protein to dimerize and phosphorylate itself, or the conformational change stimulates the phosphorylation of the monomer [47]. Here, we model the latter mechanism for autophosphorylation as a single step with rate constant π_1. The Michaelis-Menten constant for the dephosphorylation of X_2^* by M is K_{m3} and the association, dissociation and catalytic rate constants for this reaction are a_3, d_3 and k_3. The association and dissociation rate constants for the complex formed by X_1^* and X_2 are a_1 and d_1, the dissociation rate constant of this complex into X_1 and X_2^* is d_2, and the corresponding reverse association rate constant is a_2. The input protein X_1 is produced at a time-varying rate $k(t)$. Details of the chemical reactions of this system are shown in SI Section 5.6 eqn. (88). We use Theorems 1-3 to analyze this system as per Def. 1 by varying the total protein concentrations X_{T2} and M_T. This is done as follows.

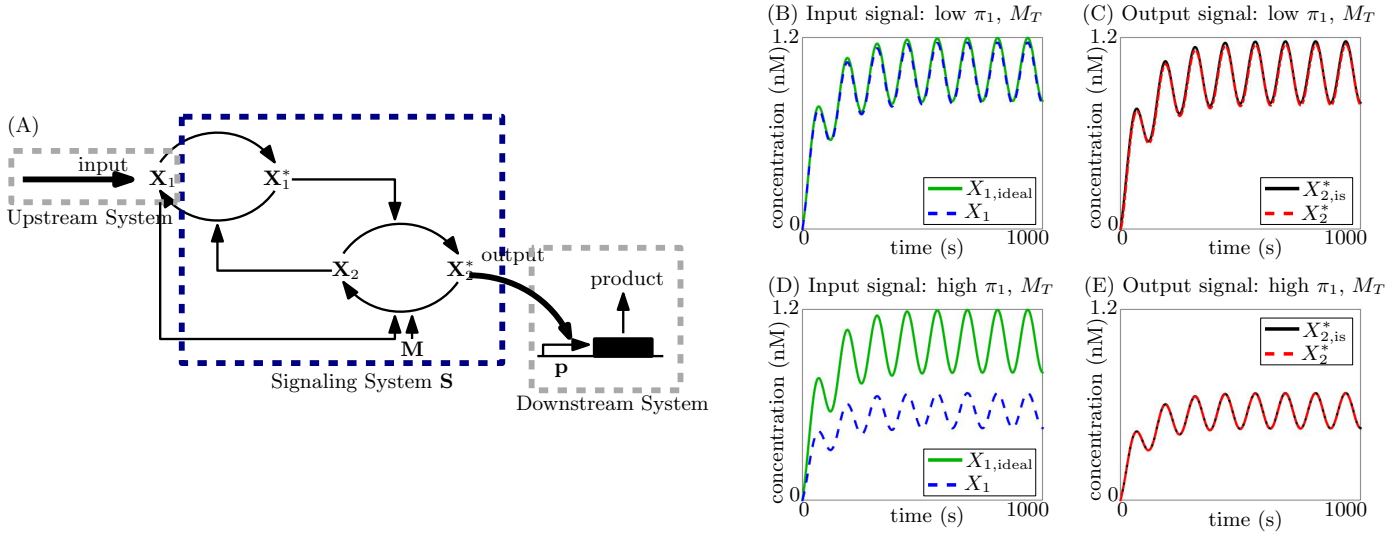


Fig 6. Attenuation of retroactivity to the output by a phosphotransfer system. (A) System with autophosphorylation followed by phosphotransfer, with input as protein X_1 which autophosphorylates to X_1^*. The phosphate group is transferred from X_1^* to X_2 by a phosphotransfer reaction, forming X_2^*, which is in turn dephosphorylated by the phosphatase M. X_2^* is the output and acts on sites p in the downstream system, which is depicted as a gene expression system here. (B)-(E) Simulation results for ODE (89) in SI Section 5.6. Common simulation parameters[1]: $k(t) = 0.01(1 + sin(0.05t)), \delta = 0.01s^{-1}, k_3 = 600s^{-1}, a_1 = a_2 = a_3 = 18nM^{-1}s^{-1}, d_1 = d_2 = d_3 = 2400s^{-1}, k_{\text{on}} = 10nM^{-1}s^{-1}, k_{\text{off}} = 10s^{-1}$, $X_{T2} = 1200nM$. (B) Effect of retroactivity to the input with low autophosphorylation rate constant π_1: for ideal input $X_{1,\text{ideal}}$, system is simulated with $\pi_1 = M_T = p_T = 0$; for actual input X_1, system is simulated with $\pi_1 = 30nM$, $M_T = 9nM$, $p_T = 100nM$. (C) Effect of retroactivity to the output with low autophosphorylation rate constant π_1: for isolated output $X_{2,\text{is}}^*$, system is simulated with $\pi_1 = 30nM$, $M_T = 9nM$, $p_T = 0$; actual output X_2^* is simulated with $\pi_1 = 30nM$, $M_T = 9nM$, $p_T = 100nM$. (D) Effect of retroactivity to the input with high autophosphorylation rate constant π_1: for ideal input $X_{1,\text{ideal}}$, system is simulated with $\pi_1 = M_T = p_T = 0$; for actual input X_1, system is simulated with $\pi_1 = 1500nM$, $M_T = 420nM$, $p_T = 100nM$. (E) Effect of retroactivity to the output with high autophosphorylation rate constant π_1: for isolated output $X_{2,\text{is}}^*$, system is simulated with $\pi_1 = 1500nM$, $M_T = 420nM$, $p_T = 0$; for actual output $X_{2,\text{is}}^*$, system is simulated with $\pi_1 = 1500nM$, $M_T = 420nM$, $p_T = 100nM$.

(i) Retroactivity to input: We make terms h_1, h_2 and h_3 from Theorem 1 small to satisfy requirement (i) of Def. 1 and have small retroactivity to the input. We find that $h_2 = 0$. Further, we find that to make h_1 and h_3 small, $\frac{2d_1a_2K}{a_1d_2X_{T2}}$, $\frac{\pi_1(d_1+d_2)}{a_1d_2X_{T2}}$, $\frac{2a_2K}{d_2}$ and $\frac{\pi_1}{d_2}$ must be small, where $K = \frac{\pi_1 K_{m3}}{k_3 M_T}$. However, not all these terms can be made smaller by varying X_{T2} and M_T alone. Thus, the retroactivity to the input, and whether or not requirement (i) is satisfied, depends on the reaction rate constants of the system, and it is not possible to tune it using total protein concentrations alone. (Mathematical details to derive these expressions are in result (i) of SI Section 5.6).

(ii) Retroactivity to output: To attenuate retroactivity to the output (requirement (ii) of Def. 1), we make $\bar{h}_1$, h_2 and $\bar{h}_3$ from Theorem 2 small. We find that $h_2 = 0$ and $\bar{h}_3 = 0$. Further we find that, to make $\bar{h}_1$ small, we must have a small

$\frac{p_T}{X_{T2}}$ and $\frac{p_T \delta}{a_3 M_T}$. Thus, to attenuate retroactivity to the output, X_{T2} and M_T must be large. (Mathematical details to derive these expressions are in result (ii) of SI Section 5.6).

(iii) Input-output relationship: Using Theorem 3, we find that the input-output relationship is $X^*_{2,\text{is}} \approx \frac{\pi_1 K_{m3}}{k_3 M_T} X_{1,\text{is}}$ when $K_{m3} \gg X^*_{2,\text{is}}$ and thus, this system can satisfy Def. 1 (iii) by tuning M_T to achieve a desired K with $m = 1$. (Mathematical details to derive these expressions are in result (i) of SI Section 5.6, eqn. (93)).

Thus, we find that the retroactivity to the input cannot be made small by changing concentrations alone. The retroactivity to the output can be attenuated by having a large X_{T2} and M_T, since these can compensate for the sequestration of X_2^* by the downstream system. This signaling system can therefore satisfy requirements (ii) and (iii) for unidirectional signal transmission. While satisfying these requirements does not increase the retroactivity to the input, thus making it possible for it to satisfy requirement (i) as well, retroactivity to the input depends on the reaction-rate parameters, in particular, on the forward reaction rate constant π_1 of autophosphorylation of X_1. If this is large, the autophosphorylation reaction applies a large reaction flux to the upstream system, thus resulting in a large retroactivity to the input. If π_1 is small, this flux is small, and thus retroactivity to the input is small. By the way we have defined cascades (as signals between stages transmitted through a kinase), any cascade containing this system would have it as a first stage. Therefore, even cascading this system with different architectures would not overcome the above limitation. These mathematical predictions can be appreciated in the simulation results shown in Figs. 6B- 6E. The result is summarized in Fig. 9C.

2.6 Single cycle with substrate input

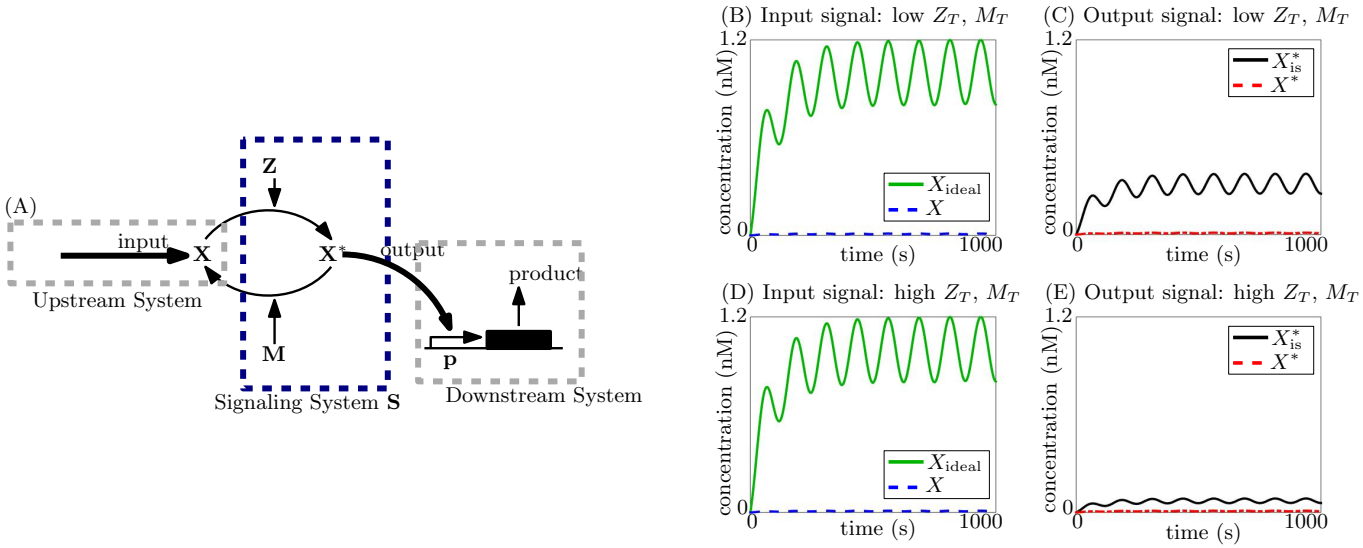


Fig 7. Inability to attenuate retroactivity to the output or impart small retroactivity to the input by single phosphorylation cycle with substrate as input. (A) Single phosphorylation cycle, with input X as the substrate: X is phosphorylated by the kinase Z to X^*, which is dephosphorylated by the phosphatase M back to X. X^* is the output and acts as a transcription factor for the promoter sites p in the downstream system. (B)-(E) Simulation results for ODE (98) in SI Section 5.7. Common simulation parameters[1]: $k(t) = 0.01(1 + sin(0.05t)), \delta = 0.01 s^{-1}, k_1 = k_2 = 600 s^{-1}, a_1 = a_2 = 18 nM^{-1} s^{-1}, d_1 = d_2 = 2400 s^{-1}, k_{\text{on}} = 10 nM^{-1} s^{-1}, k_{\text{off}} = 10 s^{-1}$. (B) Effect of retroactivity to the input with low kinase concentration Z_T: for ideal input X_{ideal}, system is simulated with $Z_T = M_T = p_T = 0$; for actual input X, system is simulated with $Z_T = M_T = p_T = 100nM$. (C) Effect of retroactivity to the output with low kinase concentration Z_T: for isolated output X^*_{is}, system is simulated with $Z_T = M_T = 100nM$, $p_T = 0$; for actual output X^*, system is simulated with $Z_T = M_T = p_T = 100nM$. (D) Effect of retroactivity to the input with high kinase concentration Z_T: for ideal for ideal input X_{ideal}, system is simulated with $Z_T = M_T = p_T = 0$; for actual input X, system is simulated with $Z_T = M_T = 1000nM$, $p_T = 100nM$. (E) Effect of retroactivity to the output with high kinase concentration Z_T: for isolated output X^*_{is}, system is simulated with $Z_T = M_T = 1000nM$, $p_T = 0$; for actual output X^*, system is simulated with $Z_T = M_T = 1000nM$, $p_T = 100nM$.

Here, we consider a single phosphorylation cycle where the input signal U of Fig. 1A is X, the concentration of the substrate X, and the output signal Y is X^*, the concentration of the phosphorylated substrate. We consider this system motivated by the various transcription factors that undergo phosphorylation before activating or repressing their targets, such as the transcriptional activator NRI in the *E. Coli* nitrogen assimilation system [48]. However, to the best of our knowledge, based on our literature review, signals are more commonly transmitted through kinases, as opposed to being transmitted by the substrates of phosphorylations. Since these are less represented than the others in natural systems, we ask whether they have any disadvantage for unidirectional transmission, and in fact they do. Note that the system analyzed in Section 2.5 is a system that takes as input a kinase that undergoes autophosphorylation before donating the phosphate group, and is not the same as the system considered here, where the input is a substrate of enzymatic phosphorylation.

The signaling system we consider, along with the upstream and downstream systems, is shown in Fig. 7A. The input protein X is produced at a time-varying rate $k(t)$. It is phosphorylated by kinase Z to the output protein X*, which is in turn dephosphorylated by phosphatase M. X* then acts as a transcription factor for the promoter sites in the downstream system. All the species in the system decay with rate constant δ. The total concentration of promoters in the downstream system is p_T. The total kinase and phosphatase concentrations are Z_T and M_T, respectively, which are the parameters of the system we vary. The Michaelis-Menten constants of the phosphorylation and dephosphorylation reactions are K_{m1} and K_{m2}, and the catalytic rate constants are k_1 and k_2. The chemical reactions of this system are shown in eqn. (97) in SI Section 5.7. Using Theorems 1, 2 and 3, we analyze if this system can transmit a unidirectional signal according to Definition 1 by varying Z_T and M_T. This is done as follows.

(i) Retroactivity to the input: As before, we seek to minimize retroactivity to the input to satisfy requirement (i) of Def. 1 using Theorem 1. However, we find that the terms h_1, h_2 and h_3 cannot be made small by changing Z_T and M_T, and therefore, retroactivity to the input cannot be made small by tuning these parameters. (Mathematical details to derive these expressions are in result (i) of SI Section 5.7).

(ii) Retroactivity to the output: Similarly, we seek to attenuate retroactivity to the output and satisfy requirement (ii) of Def. 1 using Theorem 2. However, we find that $\bar{h}_1$ and h_2 cannot be made small by varying Z_T and M_T. Thus, retroactivity to the output cannot be attenuated by tuning these parameters. (Mathematical details to derive these expressions are in result (ii) of SI Section 5.7).

(iii) Input-output relationship: Using the expression in Theorem 3, we find that the input-output relationship is linear with gain $K = \left(\frac{\frac{k_1 Z_T}{K_{m1}}}{\frac{k_2 M_T}{K_{m2}} + \delta} \right)$ when $K_{m1}, K_{m2} \gg X$, that is:

$$X^*_{is}(t) \approx K X_{is}(t). \tag{2}$$

The input-output relationship is thus linear, i.e., $m = 1$, and K can be tuned by varying Z_T and M_T. The system thus satisfies requirement (iii) of Def. 1. (Mathematical details to derive these expressions are in result (iii) of SI Section 5.7, eqn. (105)).

Thus, we find that a signaling system composed of a single phosphorylation cycle with substrate as input cannot transmit a unidirectional signal, since it can neither make retroactivity to the input small nor attenuate retroactivity to the output. This is because, the same protein X is the input (when unmodified) and the output (when phosphorylated). Thus, when X undergoes phosphorylation, the concentration of input X is reduced by conversion to X^*, thus applying a large retroactivity to the input. Now, when X* is sequestered by the downstream system, this results in a large flux to both X and X*, and thus the retroactivity to the output is also large. Cascading such a system would also not enhance its ability to transmit unidirectional signals: if the system were used as the first stage to a cascade, it would apply a large retroactivity to the input for the aforementioned reasons. The way we have defined cascades above, with non-initial stages receiving their input via a kinase, this system cannot be the second stage of a cascade since it takes its input in the form of the substrate. These results are demonstrated in the simulation results shown in Fig. 7B-7E and summarized in Fig. 9F.

2.7 Double cycle with substrate input

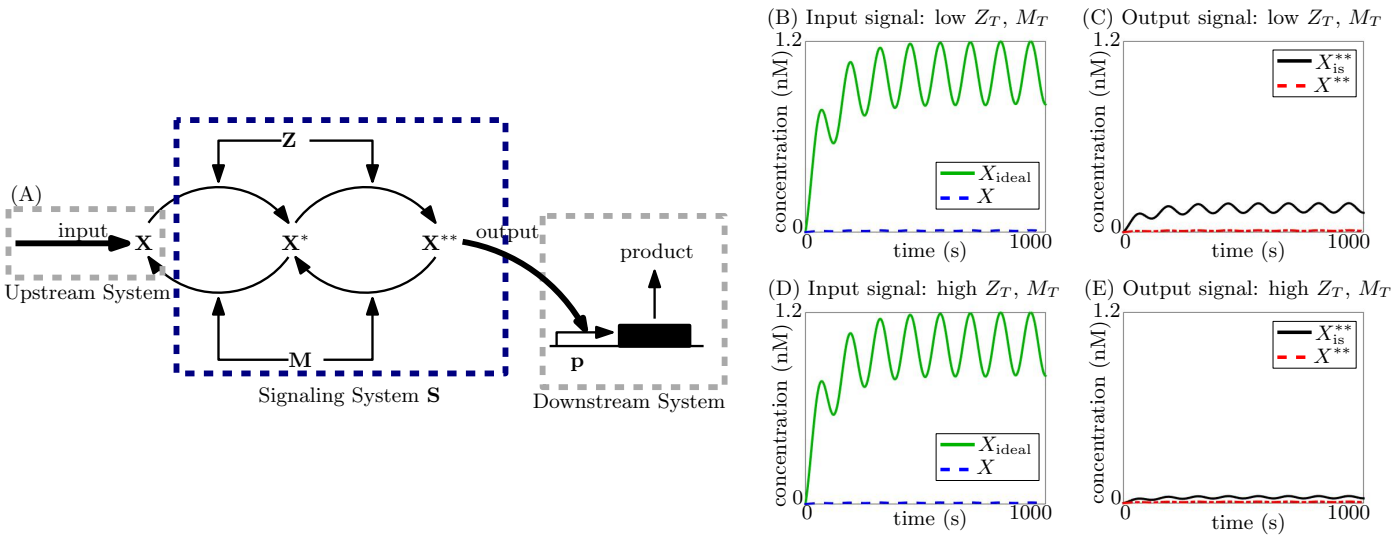


Fig 8. Inability to attenuate retroactivity to the output or impart small retroactivity to the input by double phosphorylation cycle with substrate as input. (A) Double phosphorylation cycle, with input X as the substrate: X is phosphorylated twice by the kinase K to X^* and X^{**}, which are in turn dephosphorylated by the phosphatase M. X^{**} is the output and acts on sites p in the downstream system, which is depicted as a gene expression system here. (B)-(E) Simulation results for ODE (98) in SI Section 5.8. Common simulation parameters[1]: $k(t) = 0.01(1 + sin(0.05t)), \delta = 0.01s^{-1}, k_1 = k_2 = k_3 = k_4 = 600s^{-1}, a_1 = a_2 = a_3 = a_4 = 18nM^{-1}s^{-1}, d_1 = d_2 = d_3 = d_4 = 2400s^{-1}, k_{\text{on}} = 10nM^{-1}s^{-1}, k_{\text{off}} = 10s^{-1}$. (B) Effect of retroactivity to the input with low kinase concentration: for ideal input X_{ideal}, system is simulated with $Z_T = M_T = p_T = 0$; for actual input X, system is simulated with $Z_T = M_T = 150nM$, $p_T = 100nM$. (C) Effect of retroactivity to the output with low kinase concentration: for isolated output X^{**}_{is}, system is simulated with $Z_T = M_T = 100nM$, $p_T = 0$; for actual output X^{**}, system is simulated with $Z_T = M_T = 150nM$, $p_T = 100nM$. (D) Effect of retroactivity to the input with high kinase concentration: for ideal for ideal input X_{ideal}, system is simulated with $Z_T = M_T = p_T = 0$; for actual input X, system is simulated with $Z_T = M_T = 1000nM$, $p_T = 100nM$. (E) Effect of retroactivity to the output with high kinase concentration: for isolated output X^{**}_{is}, system is simulated with $Z_T = M_T = 1000nM$, $p_T = 0$; for actual output X^{**}, system is simulated with $Z_T = M_T = 1000nM$, $p_T = 100nM$.

Finally, we consider a double phosphorylation cycle with input signal U of Fig. 1A as the concentration of the substrate, X, and the output signal Y as the concentration of the doubly phosphorylated substrate, X^{**}. Similar to the single phosphorylation cycle, we consider this system to model cases where the input species undergoes double phosphorylation before acting on its downstream targets, such as transcription factor FKHRL1, which is phosphorylated by Akt at its T23 and S253 sites [49]. In this system, the signal is transmitted by the kinase Akt and not the substrate. Based on our literature review, we have not found systems where the signal is transmitted by the substrate in such an architecture. We therefore consider this architecture to test whether it has a disadvantage for unidirectional signal transmission. The arrangement is shown in Fig. 8A. All species dilute with rate constant δ. The total concentration of promoters in the downstream system is p_T. The total concentration of kinase Z and total concentration of phosphatase M are Z_T and M_T, respectively. The input X is produced at a time-varying rate $k(t)$. Using Theorems 1, 2 and 3, we vary Z_T and M_T to investigate if this system can transmit unidirectional signals according to Def. 1. This is done as follows:

(i) Retroactivity to the input: Evaluating the terms in Theorem 1, h_1 and h_2 cannot be made small by tuning Z_T and M_T, and thus, requirement (i) of Def. 1 is not satisfied. (Mathematical details to derive these expressions are in result (i) of SI Section 5.8).

(ii) Retroactivity to the output: Evaluating the terms in Theorem 2, we find that $\bar{h}_1$ and h_2 cannot be made small by tuning Z_T and M_T. Thus, requirement (ii) of Def. 1 is not satisfied. (Mathematical details to derive these expressions are in result (ii) of SI Section 5.8).

(iii) Input-output relationship: Using Theorem 3, we find that $X^{**}_{is}(t) \approx KX_{is}(t)$ for $t \in [t_b, t_f]$ for large Michaelis-Menten constants, where K can be tuned by tuning the total kinase and phosphatase concentrations Z_T and

M_T. Thus, the system satisfies requirement (iii) of Def. 1 with $m = 1$ and a desired K. (Mathematical details to derive these expressions are in result (iii) of SI Section 5.8, eqn. (119)).

Thus, similar to the single cycle with substrate as input, the double cycle with substrate as input provides a linear input-output relationship but is not able to impart a small retroactivity to the input, nor is it able to attenuate retroactivity to the output, even upon cascading with other systems. These properties are shown in Fig. 8B-8E, and the results are summarized in Fig. 9G.

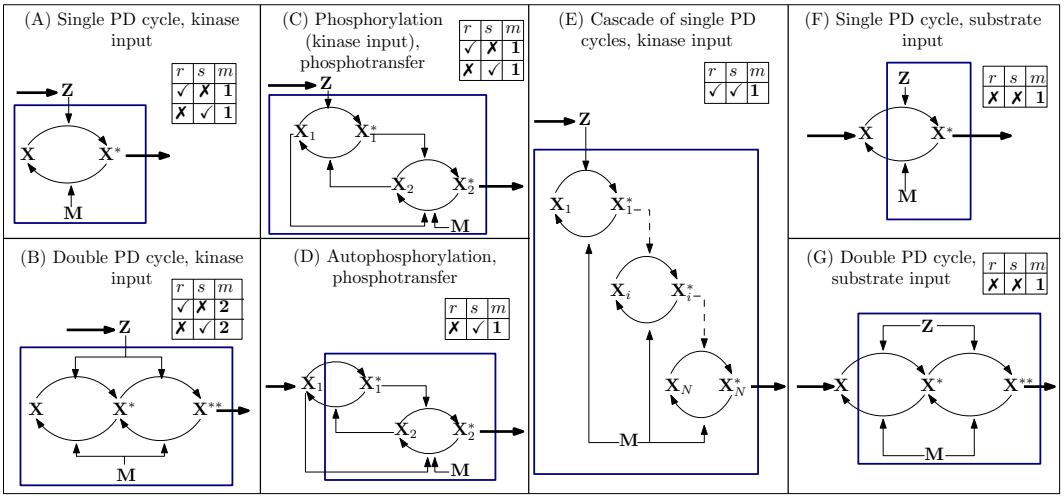


Fig 9. Table summarizing the results. For each inset table, a ✓(✗) for column r implies the system can (cannot) be designed to minimize retroactivity to the input by varying total protein concentrations, a ✓(✗) for column s implies the system can (cannot) be designed to attenuate retroactivity to the output by varying total protein concentrations, column m describes the input-output relationship of the system with m as described in Def. 1(iii). Inset tables with two rows imply that one of the two rows can be achieved for a set of values for the design parameters: thus, the two rows for systems (A), (B) and (C) show the trade-off between the ability to minimize retroactivity to the input (first row) and the ability to attenuate retroactivity to the output (second row). Note that this trade-off is overcome by the cascade (E).

3 Discussions

The goal of this work was to identify signaling architectures that can overcome retroactivity and thus allow the transmission of unidirectional signals. To achieve this, we have provided analytical expressions for retroactivity to the input and output of a general signaling system composed of reactions such as phosphorylation-dephosphorylation and phosphotransfer with a relatively slow input. We have then considered different signaling architectures, shown in Fig. 9, and have used these expressions to determine whether they have the ability to minimize retroactivity to the input and attenuate retroactivity to the output. We have found that tuning the total protein concentrations of cascaded architectures that transmit information via kinases allows them to transmit unidirectional signals. However, tuning the total protein concentrations of architectures with a substrate a input does not achieve the desired result even when cascaded.

We analyzed an architecture composed of a double phosphorylation cycle and an architecture composed of a phosphotransfer system whose phosphate donor undergoes phosphorylation, both transmitting information from an input kinase (Figs. 9B, 9C) . We found that these systems show a trade-off between minimizing retroactivity to the input (which can be achieved with a low substrate concentration) and attenuating retroactivity to the output (which requires a high substrate concentration). This trade-off has been reported in the single phosphorylation cycle before, both theoretically and experimentally [33], [50]. We have further found that when such a system with low substrate concentration is cascaded upstream of another such system with high substrate concentration, this cascade can overcome the trade-off (Fig. 9E). This is because the low substrate concentration stage then interacts (directly) with the input, imparting a small retroactivity to the input, and the high substrate concentration stage interacts (directly) with the targets, attenuating retroactivity to the output. This low-high substrate concentration pattern appears in the MAPK

signaling cascade in the mature Xenopus Oocyte, where the first stage is a phosphorylation cycle with substrate concentration $3nM$ and the last two stages are double phosphorylation cycles with substrate concentration $1200nM$ [25]. This low-high pattern indicates an ability to overcome retroactivity and transmit unidirectional signals, and while this structure may serve other purposes as well, it is possible that the substrate concentration pattern has evolved to more efficiently transmit unidirectional signals.

We have thus analyzed several different architectures of signaling systems and determined which ones are able to transmit unidirectional signals, thus providing an insight into the structure and function of signaling pathways. Our analysis is based on the assumption that the input signals to the signaling system operate on timescales slower than those of fast signaling reactions. This choice is in light of evidence that PD and phosphotransfer cycles have the ability to overcome retroactivity when processing slower input signals [8], [11], [33], [34]. Further, slow signals are common in natural and synthetic systems, such as signals arising from gene expression [38], nutrient deficiency [37] and the circadian rhythm [36]. Using this timescale separation, we have derived Theorems 1 - 3, providing expressions that can be used to evaluate a signaling system's ability to transmit unidirectional signals. An open question is whether mechanisms exist that can transmit fast signals unidirectionally.

Based on our analysis, pathways that are composed of cascades (Fig. 9E) of kinase-to-kinase phosphorylation (Figs. 9A, 9B) and phosphotransfer events (Figs. 9C), are most suited to this kind of signal transduction. These are highly represented architectures in cellular signaling [8]- [13]. In contrast, architectures that do not perform as well, such as those with substrate as input, are not as highly frequent in natural systems. It has also been reported that kinase-to-kinase relationships are highly conserved evolutionarily [51], implying that upon evolution, signaling mechanisms where kinases phosphorylate other kinases are conserved. These facts lend credence to the notion that cellular signaling has been evolving to be more efficient at one-way transmission.

For graph-based methods for analyzing cellular networks [52], such as discovering functional modules based on motif-search or clustering, signaling pathway architectures that transmit unidirectional signals can then be treated as directed edges. On the contrary, analysis of signaling systems (such as those with a substrate as input) that do not demonstrate the ability to transmit unidirectional signals must take into account effects of retroactivity. In fact, retroactivity effects could result in crosstalk between different targets of the signaling system, since a change in one target would affect the others by changing the signal being transmitted through the pathway [13]. Our work provides a way to identify signaling pathways that overcome such effects. Further, it provides a library of systems that transmit unidirectional signals, which could be used in synthetic biology to connect genetic components that function on the slow timescale of gene expression, enabling modular circuit design.

4 Methods

Theorems 1, 2 and 3 are derived using results from singular perturbation theory [53] and contraction theory [54]. Details and assumptions for these are provided in SI Section 5.1.

All reactions are modeled as two step reactions. Phosphorylation and dephosphorylation reactions are modeled as Michaelis-Menten reactions, and phosphotransfer reactions are modeled as reversible, two-step reactions resulting in the transfer of the phosphate group via the formation of an intermediate complex. Based on these reactions, as well as production and decay of the various species, ODE models are created for the systems using their reaction-rate equations. These ODE models are then brought to the generalized form (1) shown in Section 2 and analyzed using Theorems 1-3. This analysis is verified using simulations of the full ODE systems run on MATLAB. The numerical ODE solver ode23s was used to run simulations for systems 2.4 and 2.5, and ode15s was used to run simulations for systems 2.2, 2.3, 2.6 and 2.7.

5 Supplementary Information

5.1 Assumptions and Proofs for Theorems 1-3

For the general system (1), we make the following Assumptions:

Assumption 1. Phosphorylation-dephosphorylation and phosphotransfer reactions typically occur at rates of the order of second^{-1} [55], [56], much faster than transcription, translation and decay, which typically occur at rates of the order of

hour^{-1} [57]. Then, $G_1 \gg 1$.

Assumption 2. Binding-unbinding reactions of the output with the promoter sites in the downstream system are much faster than transcription, translation and decay [58]. Then, $G_2 \gg 1$.

Assumption 3. The eigenvalues of $\frac{\partial(B\underline{r}+f_1)}{\partial \underline{X}}$ and $\frac{\partial s}{\partial v}$ have strictly negative real parts.

Assumption 4. There exist invertible matrices T and Q, and matrices M and P, such that $TA + MB = 0$, $Mf_1 = 0$ and $QC + PD = 0$.

Assumption 5. Let $\underline{X} = \underline{\Psi}(U, v)$ be the locally unique solution to $f_1(U, \underline{X}, S_3 v) + B\underline{r}(U, \underline{X}, S_2 v) = 0$. We assume $\underline{\Psi}(U, v)$ is Lipschitz continuous in v with Lipschitz constant L_Ψ.

Assumption 6. Let $v = \phi(\underline{X})$ be the locally unique solution to $s(\underline{X}, v) = 0$. Define the function $f(U, \underline{X}) = \underline{X} - \underline{\Psi}(U, \phi(\underline{X}))$. Then the matrix $\frac{\partial f(U,\underline{X})}{\partial \underline{X}} \in \mathbb{R}^{n\times n}$ is invertible.

Assumption 7. Let $\underline{\Gamma}(U)$ be the locally unique solution to $B\underline{r}(U, \underline{X}, S_2 v) + f_1(U, \underline{X}, S_3 v) = 0$. We assume that $\underline{\Gamma}(U)$ is Lipschitz continuous with Lipschitz constant L_Γ.

Remark 1. By definition of $\underline{\Gamma}(U)$, we have that $\underline{\Gamma}(U) = \underline{\Psi}(U, \phi(\underline{\Gamma}(U)))$, since $v = \phi(\underline{X})$ satisfies $s(\underline{X}, v) = 0$ and $\underline{X} = \underline{\Psi}(U, \underline{X})$ satisfies $f_1(U, \underline{X}, S_3 v) + B\underline{r}(U, \underline{X}, S_2 v) = 0$. If $S_2 = S_3 = 0$, $\underline{\Gamma}(U)$ is independent of v, which is denoted by $\underline{\Gamma}_{is}(U)$. Then, $\underline{\Gamma}_{is}(U) = \underline{\Psi}(U, 0)$ since $S_2 = S_3 = 0$. Thus, the difference $|\underline{\Gamma}_{is}(U) - \underline{\Gamma}(U)|$ depends on S_2 and S_3, and is zero when $S_2 = S_3 = 0$. We thus sometimes denote $\underline{\Gamma}(U)$ as $\underline{\Psi}(U, g(S_2, S_3)\phi(\underline{\Gamma}(U)))$, where $g(S_2, S_3) = 0$ if both $S_2 = S_3 = 0$. Further, since as $||S_2||$ and $||S_3||$ decrease, the dependence of $f_1(U, \underline{X}, S_3 v) + B\underline{r}(U, \underline{X}, S_2 v)$ on v decreases, by the implicit function theorem, $g(S_2, S_3)$ decreases as $||S_2||$ and $||S_3||$ decrease.

Assumption 8. The function $f_0(U, t)$ is Lipschitz continuous in U with Lipschitz constant L_0. The function $\underline{r}(U, \underline{X}, v)$ is Lipschitz continuous in $\underline{X}$ and v.

Assumption 9. The system:

$$\dot{U} = f_0(U, R\underline{\Gamma}(U), S_1\phi(\underline{\Gamma}(U)), t) + G_1 A\underline{r}(U, \underline{\Gamma}(U), S_2\phi(\underline{\Gamma}(U)))$$

is contracting [54] with parameter λ.

We now state the following result from [50]:

Lemma 1. *If the following system:*

$$\dot{x} = f(x, t)$$

is contracting with contraction rate λ, then, for the perturbed system:

$$\dot{\bar{x}} = f(\bar{x}, t) + d(\bar{x}, t),$$

where there exists a $\bar{d} \geq 0$ such that $|d(\bar{x}, t)| \leq \bar{d}$ for all $\bar{x}, t$, the difference in trajectories for the actual and perturbed system is given by:

$$|x(t) - \bar{x}(t)| \leq e^{-\lambda t}|x(0) - \bar{x}(0)| + \frac{\bar{d}}{\lambda}.$$

We state the following result, adapted from [32], for system (1):

Lemma 2. *Under Assumptions 1-4, $||\underline{X}(t) - \underline{\Psi}(U(t), v(t))|| = \mathcal{O}(\frac{1}{G_1})$ and $||v(t) - \phi(\underline{X}(t))|| = \mathcal{O}(\frac{1}{G_2})$ for $t \in [t_b, t_f]$, where $\underline{\Psi}(U, v)$ is defined in Assumption 5, $\phi(\underline{X})$ is defined in Assumption 6 and t_b is such that $t_i < t_b < t_f$ and $t_b - t_i$ decreases as G_1 and G_2 increase.*

Proof of Lemma 2. We bring the system to standard singular perturbation form, by defining $\underline{w} = Q\underline{X} + Pv$ and $z = TU + M(\underline{X} + Q^{-1}Pv)$. Under Assumption 4, we obtain the following system:

$$\begin{aligned}
&\dot{z} = Tf_0(U, R\underline{X}, S_1 v, t),\\
&\frac{1}{G_1}\dot{\underline{w}} = Q[Br(U, \underline{X}, S_2 v) + f_1(U, \underline{X}, S_3 v)],\\
&\frac{1}{G_2}\dot{v} = G_2 Ds(\underline{X}, v),\\
&\text{where: } U = T^{-1}(z - MQ^{-1}v), \underline{X} = Q^{-1}(\underline{w} - Pv).
\end{aligned} \tag{3}$$

Under Assumptions 1-3, this system is in the standard singular perturbation form with $\epsilon = \max\{\frac{1}{G_1}, \frac{1}{G_2}\}$. We define function $\underline{W}(z, v)$, such that $\underline{w} = \underline{W}$ is a solution to $(Br + f_1)(z, \underline{w}, v) = 0$ and function $V(\underline{w})$ such that $v = V$ is a solution to $s(\underline{w}, v) = 0$. Applying singular perturbation, we then have $||\underline{w}(t) - \underline{W}(z, v)|| = \mathcal{O}(\frac{1}{G_1})$ and $||v(t) - V(\underline{w})|| = \mathcal{O}(\frac{1}{G_2})$. Rewriting these expressions in terms of the original variables, we use the definitions in Assumptions 5 and 6, we have: $||\underline{X}(t) - \underline{\Psi}(U, v)|| = \mathcal{O}(\frac{1}{G_1})$ and $||v(t) - \phi(\underline{X})|| = \mathcal{O}(\frac{1}{G_2})$. ■

Lemma 3. *Under Assumptions 1-6, $||\underline{X}(t) - \underline{\Gamma}(U(t))|| = \mathcal{O}(\epsilon)$, for $t \in [t_b, t_f]$, where $\underline{\Gamma}(U)$ is defined in Remark 1.*

Proof of Lemma 3. From Lemma 2, we have:

$$\begin{aligned}
\underline{X} &= \underline{\Psi}\left(U, \phi(\underline{X}) + \mathcal{O}(\frac{1}{G_2})\right) + \mathcal{O}(\frac{1}{G_1})\\
&= \underline{\Psi}\left(U, \phi(\underline{X})\right) + \underline{\Psi}\left(U, \phi(\underline{X}) + \mathcal{O}(\frac{1}{G_2})\right) - \underline{\Psi}\left(U, \phi(\underline{X})\right) + \mathcal{O}(\frac{1}{G_1}).
\end{aligned}$$

Under Assumption 5, using the Lipschitz continuity of $\underline{\Psi}(U, v)$ we have:

$$\underline{X} \leq \underline{\Psi}\left(U, \phi(\underline{X})\right) + L_\Psi \mathcal{O}(\frac{1}{G_2}) + \mathcal{O}(\frac{1}{G_1}).$$

By definition of $\mathcal{O}$, we have:

$$\underline{X} \leq \underline{\Psi}\left(U, \phi(\underline{X})\right) + \mathcal{O}(\max(\frac{1}{G_1}, \frac{1}{G_2}) = \epsilon). \tag{4}$$

By equation (4), $f(U, \underline{U}) \leq \mathcal{O}(\epsilon)$, where the function f is defined in Assumption 4. By definition of $\underline{\Gamma}(U)$, we have $f(U, \underline{\Gamma}(U)) = \underline{\Gamma}(U) - \underline{\Psi}(U, \phi(\underline{\Gamma}(U))) = 0$. Therefore:

$$f(U, \underline{X}) - f(U, \underline{\Gamma}(U)) \leq \mathcal{O}(\epsilon).$$

Under Assumption 5, $f(U, \underline{X})$ is differentiable. Applying the Mean Value theorem [59], we have:

$$f(U, \underline{X}) - f(U, \underline{\Gamma}(U)) = (\underline{X} - \underline{\Gamma}(U))\frac{\partial f(U, \underline{X})}{\partial \underline{X}}\bigg|_{\underline{X} = \underline{c}} \leq \mathcal{O}(\epsilon).$$

Under Assumption 6, the matrix $\frac{\partial f(U, \underline{X})}{\partial \underline{X}}\Big|_{\underline{X} = \underline{c}}$ is invertible. Thus,

$$||\underline{X} - \underline{\Gamma}(U)|| = \mathcal{O}(\epsilon).$$

■

Lemma 4. *Under Assumptions 1-6, 8-9, for $t \in [t_b, t_f]$, $|U(t) - \bar{U}(t)| = \mathcal{O}(\epsilon)$ where $\bar{u}$ is such that:*

$$\dot{\bar{U}} = f_0(\bar{U}, R\underline{\Gamma}(\bar{U}), S_1\phi(\underline{\Gamma}(\bar{U})), t) + G_1 A\underline{r}(\bar{U}, \underline{\Gamma}(\bar{U}), S_2\phi(\underline{\Gamma}(\bar{U}))),\ \bar{U}(0) = U(0). \tag{5}$$

Proof of Lemma 4.

$$\begin{aligned}\dot{U} &= f_0(U, R\underline{X}, S_1 v, t) + G_1 A\underline{r}(U, \underline{X}, S_2 v)\\ &= f_0(U, R\underline{\Gamma}(U), S_1\phi(\underline{\Gamma}(U)), t) + G_1 A\underline{r}(U, \underline{\Gamma}(U), S_2\phi(\underline{\Gamma}(U))) + \mathcal{O}(\epsilon),\end{aligned}$$

by Lemmas 2 and 3, since the functions f_0 and $\underline{r}$ are Lipschitz continuous under Assumption 8. Applying Lemma 1 to this system under Assumption 9, we have $|U(t) - \bar{U}(t)| = \mathcal{O}(\epsilon)$. ■

Proof of Theorem 1. By definition of U_{ideal}, we have from (1):

$$\dot{U}_{\text{ideal}} = f_0(U_{\text{ideal}}, 0, 0, t),\ U_{\text{ideal}}(0) = U(0).$$

We define $\bar{U}$ such that its dynamics are given by (5), that is:

$$\dot{\bar{U}} = f_0(\bar{U}, R\underline{\Gamma}(\bar{U}), S_1\phi(\underline{\Gamma}(\bar{U})), t) + G_1 A\underline{r}(\bar{U}, \underline{\Gamma}(\bar{U}), S_2\phi(\underline{\Gamma}(\bar{U}))),\ \bar{U}(0) = U(0). \tag{6}$$

By the Lipschitz continuity of f_0 under Assumption 8, we have:

$$f_0(\bar{U}, R\underline{\Gamma}(\bar{U}), S_1\phi(\underline{\Gamma}(\bar{U})), t) = f_0(\bar{U}, 0, 0, t) + h(\bar{U}), \tag{7}$$

where $|h(\bar{U})| \leq L_0|R\underline{\Gamma}(\bar{U})| + L_0|S_1\phi(\underline{\Gamma}(\bar{U}))|$. Thus, $|h(\bar{U})| \leq h_1 + h_2$.

Further define $z = TU + M\underline{X} + MQ^{-1}Pv$. Then,

$$\dot{z} = T\dot{U} + M\dot{\underline{X}} + MQ^{-1}P\dot{v} = Tf_0(U, R\underline{X}, S_1 v, t)$$

from eqns. (1). Using the expression of $\dot{U}$ from (1), we then see that

$$G_1 Ar(U, \underline{X}, S_2 v) = -T^{-1}M\dot{\underline{X}} - T^{-1}MQ^{-1}P\dot{v}.$$

By Lemma 2 we have $v = \phi(\underline{X}) + \mathcal{O}(\frac{1}{G_2})$ for $t \in [t_b, t_f]$. By Lemma 3we have $\underline{X} = \underline{\Gamma}(U) + \mathcal{O}(\epsilon)$ for $t \in [t_b, t_f]$. Thus,

$$\dot{\underline{X}} = \frac{\partial\underline{\Gamma}(U)}{\partial U}\dot{U}, \dot{v} = \left.\frac{\partial\phi(\underline{X})}{\partial\underline{X}}\right|_{\underline{X}=\underline{\Gamma}}\frac{\partial\underline{\Gamma}(U)}{\partial U}\dot{U} \text{ for } t \in [t_b, t_f].$$

This implies that

$$G_1 Ar(U, \underline{X}, S_2 v) = -T^{-1}M\frac{\partial\underline{\Gamma}(U)}{\partial U}\dot{U} - T^{-1}MQ^{-1}P\left.\frac{\partial\phi(\underline{X})}{\partial\underline{X}}\right|_{\underline{X}=\underline{\Gamma}}\frac{\partial\underline{\Gamma}(U)}{\partial U}\dot{U} \text{ for } t \in [t_b, t_f].$$

Then, under Assumption 8, due to the Lipschitz continuity of $\underline{r}$ and Lemmas 2 and 3,

$$G_1 Ar(U, \underline{\Gamma}(U), S_2\phi(\underline{\Gamma}(U))) = -T^{-1}M\frac{\partial\underline{\Gamma}(U)}{\partial U}\dot{U} - T^{-1}MQ^{-1}P\left.\frac{\partial\phi(\underline{X})}{\partial\underline{X}}\right|_{\underline{X}=\underline{\Gamma}}\frac{\partial\underline{\Gamma}(U)}{\partial U}\dot{U} + \mathcal{O}(\epsilon),$$

for $t \in [t_b, t_f]$. Changing variables does not change the result, i.e., we define $q(\bar{U})$ such that

$$\begin{aligned}q(\bar{U}) &= G_1 Ar(\bar{U}, \underline{\Gamma}(\bar{U}), S_2\phi(\underline{\Gamma}(\bar{U})))\\ &= -T^{-1}M\frac{\partial\underline{\Gamma}(\bar{U})}{\partial\bar{U}}\dot{\bar{U}} - T^{-1}MQ^{-1}P\left.\frac{\partial\phi(\underline{X})}{\partial\underline{X}}\right|_{\underline{X}=\underline{\Gamma}}\frac{\partial\underline{\Gamma}(\bar{U})}{\partial\bar{U}}\dot{\bar{U}} + \mathcal{O}(\epsilon)\end{aligned}$$

. From the definition of h_3 in Theorem 1, we have that $|q(\bar{U})| \leq h_3 + \mathcal{O}(\epsilon)$. Thus, the dynamics of $\bar{U}$ as given by eqn. (6) can be rewritten using eqn. (7) and $q(\bar{U}) = G_1 Ar(\bar{U}, \underline{\Gamma}(\bar{U}), S_2\phi(\underline{\Gamma}(\bar{U})))$ as:

$$\dot{\bar{U}} = f_0(\bar{U}, 0, 0, t) + h(\bar{U}) + q(\bar{U}).$$

Using Lemma 1 we have that

$$|U_{\text{ideal}}(t) - \bar{U}(t)| \leq \frac{h_1 + h_2 + h_3 + \mathcal{O}(\epsilon)}{\lambda},$$

for $t \in [t_b, t_f]$. From the triangle inequality, we know that $|U_{\text{ideal}}(t) - U(t)| \leq |U_{\text{ideal}}(t) - \bar{U}(t)| + |\bar{U}(t) - U(t)|$. Using Theorem 4, we have:

$$|U_{\text{ideal}}(t) - U(t)| \leq \frac{h_1 + h_2 + h_3}{\lambda} + \mathcal{O}(\epsilon), \quad \text{for } t \in [t_b, t_f].$$

■

Proof of Theorem 2. By definition, $Y(t) = I\underline{X}(t)$. Under Lemma 3, this implies that $Y(t) = I\underline{\Gamma}(U(t)) + \mathcal{O}(\epsilon)$. The isolated output is then $Y_{\text{is}}(t) = I\underline{\Gamma}_{\text{is}}(U_{\text{is}}(t)) + \mathcal{O}(\epsilon)$. Thus,

$$\begin{aligned} |Y_{\text{is}}(t) - Y(t)| &= ||I||\,|\underline{\Gamma}(U) - \underline{\Gamma}_{\text{is}}(U_{\text{is}})| + \mathcal{O}(\epsilon) \\ &\leq ||I||\,|\underline{\Gamma}(U) - \underline{\Gamma}_{\text{is}}(U)| + ||I||\,|\underline{\Gamma}_{\text{is}}(U) - \underline{\Gamma}_{\text{is}}(U_{\text{is}})| + \mathcal{O}(\epsilon), \end{aligned} \tag{8}$$

by the triangle inequality. By definition, as seen in Remark 1, $\underline{\Gamma}(U) = \underline{\Psi}(U, g(S_2, S_3)\phi(\underline{\Gamma}(U)))$, where $g(S_2, S_3) = 0$ for $S_2 = S_3 = 0$. Also seen in Remark 1, $\underline{\Gamma}_{\text{is}}(U) = \underline{\Psi}(U, 0)$. Then, under Assumption 5,

$$|\underline{\Gamma}(U) - \underline{\Gamma}_{\text{is}}(U)| \leq L_\Psi\,|g(S_2, S_3)\phi(\underline{\Gamma}(U))| \leq \bar{d}_1. \tag{9}$$

Under Assumption 7,

$$|\underline{\Gamma}_{\text{is}}(U) - \underline{\Gamma}_{\text{is}}(U_{\text{is}})| \leq L_\gamma |U - U_{\text{is}}|. \tag{10}$$

We now define $z = TU + M\underline{X} + MQ^{-1}Pv$. Then, from eqn. (1),

$$\dot{z} = T\dot{U} + M\dot{\underline{X}} + MQ^{-1}P\dot{v} = Tf_0(U, R\underline{X}, S_1 v, t).$$

Then,

$$\dot{U} = f_0(U, R\underline{X}, S_1 v, t) - T^{-1}M\dot{\underline{X}} - T^{-1}MQ^{-1}P\dot{v}.$$

Comparing the equation above to eqns. (1) we have

$$G_1 A\underline{r}(U, \underline{X}, S_2 v) = -T^{-1}M\dot{\underline{X}} - T^{-1}MQ^{-1}P\dot{v}.$$

Thus we have that

$$\begin{aligned} G_1 A\underline{r}(U, \underline{\Gamma}(U), S_2\phi(\underline{\Gamma}(U)) &= -T^{-1}M\dot{\underline{\Gamma}}(U) - T^{-1}MQ^{-1}P\dot{\phi}\underline{\Gamma}(U) \\ &= -T^{-1}M\frac{\partial\underline{\Gamma}(U)}{\partial U}\dot{U} - T^{-1}MQ^{-1}P\frac{\partial\phi}{\partial\underline{X}}\bigg|_{\underline{X}=\underline{\Gamma}}\frac{\partial\underline{\Gamma}(U)}{\partial U}\dot{U}. \end{aligned}$$

Thus, defining $\bar{U}$ as in eqn. (5), we have:

$$\dot{\bar{U}} = f_0(\bar{U}, R\underline{\Gamma}(\bar{U}), S_1\phi(\underline{\Gamma}(\bar{U})), t) - T^{-1}M\frac{\partial\underline{\Gamma}(\bar{U})}{\partial\bar{U}}\dot{\bar{U}} - T^{-1}MQ^{-1}P\frac{\partial\phi}{\partial\underline{X}}\bigg|_{\underline{X}=\underline{\Gamma}}\frac{\partial\underline{\Gamma}(\bar{U})}{\partial\bar{U}}\dot{\bar{U}}.$$

By the Lipschitz continuity of f_0 under Assumption 8, this can be written as:

$$\dot{\bar{U}} = f_0(\bar{U}, R\underline{\Gamma}(\bar{U}), 0, t) - T^{-1}M\frac{\partial\underline{\Gamma}(\bar{U})}{\partial\bar{U}}\dot{\bar{U}} + q_2(\bar{U}) - g_2(\bar{U}), \tag{11}$$

where $|q_2(\bar{U})| \leq L_0|S_1\phi(\underline{\Gamma}(\bar{U}))|$ for all $\bar{U}$. Thus, from the definition of h_2 in Theorem 2, we have that $|q_2(\bar{U})| \leq h_2$. Further, we have

$$|g_2(U)| = \left|\left(T^{-1}MQ^{-1}P\frac{\partial\phi}{\partial\underline{X}}\bigg|_{\underline{X}=\underline{\Gamma}}\frac{\partial\underline{\Gamma}(\bar{U})}{\partial\bar{U}}\right)\dot{\bar{U}}\right| \leq \bar{h}_3, \text{ for all } \bar{U}, t \in [t_b, t_f].$$

Since $\dot{U} = f_0(U, R\underline{X}, S_1 v, t) - T^{-1}M\dot{\underline{X}} - T^{-1}MQ^{-1}P\dot{v}$, the isolated input dynamics are by definition: $\dot{U}_{is} = f_0(U, R\underline{X}, 0, t) - T^{-1}M\dot{\underline{X}}$. By Lemma 3 and under Assumption 8, this can be written as:

$$\dot{U}_{is} = f_0(U, R\underline{\Gamma}(U_{\text{is}}), 0, t) - T^{-1}M\frac{\partial\underline{\Gamma}(U_{\text{is}})}{\partial U_{\text{is}}}\dot{U}_{\text{is}}. \tag{12}$$

Applying Lemma 1 to systems (11) and (12) under Assumption 9, we have: $|\bar{U}(t) - U_{\text{is}}(t)| \leq \frac{h_2 + \bar{h}_3}{\lambda}$. By the triangle inequality and Lemma 4,

$$|U(t) - U_{\text{is}}(t)| \leq |U(t) - \bar{U}(t)| + |\bar{U}(t) - U_{\text{is}}(t)| \leq \frac{h_2 + \bar{h}_3}{\lambda} + \mathcal{O}(\epsilon). \tag{13}$$

Using (8), (9), (10) and (13), we obtain the desired result. ■

Proof of Theorem 3. From Remark 1, we see that $\underline{\Gamma}_{is}(U_{is}) = \underline{\Psi}(U_{\text{is}}, 0)$. From Lemma 2, we have $||\underline{X}_{\text{is}}(t) - \underline{\Psi}(U_{\text{is}}, 0)|| = \mathcal{O}(\epsilon)$. Thus, for $y_{is} = I\underline{X}_{\text{is}}$, we have

$$||Y_{\text{is}} - I\underline{\Gamma}_{\text{is}}(U_{\text{is}})|| = \mathcal{O}(\epsilon)$$

. ■

5.2 Single cycle with kinase input

The reactions for this system are:

$$Z \underset{k(t)}{\overset{\delta}{\rightleftharpoons}} \phi, \qquad X \underset{k_X}{\overset{\delta}{\rightleftharpoons}} \phi, \tag{14}$$

$$M \underset{k_M}{\overset{\delta}{\rightleftharpoons}} \phi, \qquad C_1, C_2, X^*, C \xrightarrow{\delta} \phi, \tag{15}$$

$$Z + X \underset{d_1}{\overset{a_1}{\rightleftharpoons}} C_1 \xrightarrow{k_1} X^* + Z, \qquad K_{m1} = \frac{d_1 + k_1}{a_1}, \tag{16}$$

$$X^* + M \underset{d_2}{\overset{a_2}{\rightleftharpoons}} C_2 \xrightarrow{k_2} M + X, \qquad K_{m2} = \frac{d_2 + k_2}{a_2}, \tag{17}$$

$$X^* + p \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} C. \tag{18}$$

Using reaction-rate equations, and the conservation law for the promoter $p_T = p + C$, the ODEs for this system are then:

$$\begin{aligned}
\frac{dZ}{dt} &= k(t) - \delta Z - a_1 ZX + (d_1 + k_1)C_1, & Z(0) &= 0,\\
\frac{dX}{dt} &= k_X - \delta X - a_1 ZX + d_1 C_1 + k_2 C_2, & X(0) &= \frac{k_X}{\delta} = X_T,\\
\frac{dM}{dt} &= k_M - \delta M - a_2 X^* M + (d_2 + k_2)C_2, & M(0) &= \frac{k_M}{\delta} = M_T,\\
\frac{dC_1}{dt} &= a_1 ZX - (d_1 + k_1)C_1 - \delta C_1, & C_1(0) &= 0,\\
\frac{dC_2}{dt} &= a_2 X^* M - (d_2 + k_2)C_2 - \delta C_2, & C_2(0) &= 0,\\
\frac{dX^*}{dt} &= k_1 C_1 - a_2 X^* M + d_2 C_2 - \delta X^* - k_{\text{on}} X^*(p_T - C) + k_{\text{off}} C, & X^*(0) &= 0,\\
\frac{dC}{dt} &= k_{\text{on}} X^*(p_{\text{T}} - C) - k_{\text{off}} C - \delta C, & C(0) &= 0.
\end{aligned} \tag{19}$$

For the system defined by (19), let $M_T = M + C_2$. Then the dynamics of M_T are $\dot{M}_T = k_M - \delta M_T, M_T(0) = \frac{k_M}{\delta}$. This gives a constant $M_T(t) = \frac{k_M}{\delta}$. The variable $M = M_T - C_2$ is then eliminated from the system. Similarly, we define $X_T = X + C_1 + C_2 + X^* + C$, whose dynamics become $\dot{X}_T = k_X - \delta X_T$, $X_T(0) = \frac{k_X}{\delta}$. Thus, $X_T(t) = \frac{k_X}{\delta}$ is a constant. The variable $X = X_T - C_1 - C_2 - X^* - C$ can then be eliminated from the system. Further, we non-dimensionalize C with respect to p_T, such that $c = \frac{C}{p_T}$. The system thus reduces to:

$$\begin{aligned}
\frac{dZ}{dt} &= k(t) - \delta Z - a_1 Z(X_T - C_1 - C_2 - X^* - p_T c) + (d_1 + k_1)C_1, & Z(0) &= 0,\\
\frac{dC_1}{dt} &= a_1 Z(X_T - C_1 - C_2 - X^* - p_T c) - (d_1 + k_1)C_1 - \delta C_1, & C_1(0) &= 0,\\
\frac{dC_2}{dt} &= a_2 X^*(M_T - C_2) - (d_2 + k_2)C_2 - \delta C_2, & C_2(0) &= 0,\\
\frac{dX^*}{dt} &= k_1 C_1 - a_2 X^*(M_T - C_2) + d_2 C_2 - \delta X^* - k_{\text{on}} X^* p_T(1 - c) + k_{\text{off}} p_T c, & X^*(0) &= 0,\\
\frac{dc}{dt} &= k_{\text{on}} X^*(1 - c) - k_{\text{off}} c - \delta c, & c(0) &= 0.
\end{aligned} \tag{20}$$

U	Z	v	c
$\underline{X}$	$\begin{bmatrix} C_1 & C_2 & X^* \end{bmatrix}^T_{3\times1}$	$Y,\ I$	$X^*, \begin{bmatrix} 0 & 0 & 1 \end{bmatrix}_{1\times3}$
G_1	$\max\left\{\frac{a_1X_T}{\delta}, \frac{d_1}{\delta}, \frac{k_1}{\delta}, \frac{a_2X_T}{\delta}, \frac{d_2}{\delta}, \frac{k_2}{\delta}, \right\}$	G_2	$\max\left\{\frac{k_{\text{on}}p_T}{\delta}, \frac{k_{\text{off}}}{\delta}\right\}$
$f_0(U, R\underline{X}, S_1v, t)$	$k(t) - \delta Z - \delta C_1$	$s(\underline{X}, v)$	$\frac{1}{G_2}\left(k_{\text{on}}X^*(1-c) - k_{\text{off}}c - \delta c\right)$
$\underline{r}(U, \underline{X}, S_2v)$	$\frac{1}{G_1}\begin{bmatrix} -a_1ZX_T(1 - \frac{X^*}{X_T} - \frac{C_1}{X_T} - \frac{C_2}{X_T} - \frac{p_T}{X_T}c) + (d_1+k_1)C_1 + \delta C_1 \end{bmatrix}_{1\times1}$		
$f_1(u, \underline{x}, S_3v)$	$\frac{1}{G_1}\begin{bmatrix} 0 \\ a_2X^*(M_T - C_2) - (d_2+k_2)C_2 - \delta C_2 \\ k_1C_1 - a_2M_T(X^* + \frac{\delta p_T}{a_2M_T}c) + a_2X^*C_2 + d_2C_2 - \delta X^* \end{bmatrix}_{3\times1}$		
A	1	D	1
B	$\begin{bmatrix} -1 & 0 & 0 \end{bmatrix}^T_{3\times1}$	C	$\begin{bmatrix} 0 & 0 & -p_T \end{bmatrix}^T_{3\times1}$
R	$\begin{bmatrix} 1 & 0 & 0 \end{bmatrix}_{1\times3}$	S_1	0
S_2	$\frac{p_T}{X_T}$	S_3	$\frac{\delta p_T}{a_2M_T}$
T	1	M	$\begin{bmatrix} 1 & 0 & 0 \end{bmatrix}_{1\times3}$
Q	$\mathbb{I}_{3\times3}$	P	$\begin{bmatrix} 0 & 0 & p_T \end{bmatrix}^T_{3\times1}$

Table 1. System variables, functions and matrices for a double phosphorylation cycle with the kinase for both cycles as input brought to form (1).

Based on eqns. (20), we bring the system to form (1) as shown in Table 1.

We now solve for $\underline{\Psi}$, ϕ and $\underline{\Gamma}$ as defined by Assumptions 5, 6 and 7.

Solving for $\underline{X} = \underline{\Psi}(U, v)$ setting $(Br + f_1)_{3\times1} = 0$, we have:

$$(Br + f_1)_2 = 0 \implies a_2X^*(M_T - C_2) = ((d_2 + k_2) + \delta)\, C_2.$$
Under Assumption 1, $(d_2 + k_2) \gg \delta$.
Then, $M_TX^* - X^*C_2 \approx K_{m2}C_2$. (21)
If $K_{m2} \gg X^*, C_2 \approx \dfrac{X^*M_T}{K_{m2}}$.

$$(Br + f_1)_2 + (Br + f_1)_3 = 0 \implies (k_1 - \delta)\, C_1 - (k_2 - \delta)\, C_2 = 0.$$
Under Assumption 1, $k_1, k_2 \gg \delta$. Then, $C_1 = \dfrac{k_2}{k_1}C_2 \approx \dfrac{k_2}{k_1}\dfrac{X^*M_T}{K_{m2}}$. (22)

$$(Br + f_1)_1 = 0 \implies \frac{a_1X_T}{\delta}ZX_T(1 - \frac{X^*}{X_T} - \frac{C_1}{X_T} - \frac{C_2}{X_T} - \frac{p_T}{X_T}c) = (d_1 + k_1 + \delta)\, C_1.$$
Under Assumption 1, $d_1 + k_1 \gg \delta$.Using (21), (22):
$$ZX_T(1 - \frac{X^*}{X_T} - (1 + \frac{k_2}{k_1})\frac{X^*M_T}{X_TK_{m2}} - \frac{p_T}{X_T}c) \approx K_{m1}\frac{k_2}{k_1}\frac{X^*M_T}{X_TK_{m2}}.$$
Thus, $X^* \approx \dfrac{ZX_T(1 - \frac{p_T}{X_T}c)}{\left(\frac{k_2K_{m1}}{k_1K_{m2}}M_T\right) + \left(1 + (1 + \frac{k_2}{k_1})\frac{M_T}{K_{m2}}\right)Z}$.

Note that as the input Z becomes very large, the output X^* saturates to $\frac{1}{1+(1+\frac{k_2}{k_1})\frac{M_T}{K_{m2}}}$. Since this violates condition (iii) of Def. 1, we must have $K_{m1} \gg Z$ and $\frac{k_2 K_{m1}}{k_1 K_{m2}} M_T \gg Z$. This gives a range of input z for which condition (iii) of Def. 1 is satisfied. Once the input increases so that $K_{m1} \gg Z$ and $\frac{k_2 K_{m1}}{k_1 K_{m2}} M_T \gg Z$ are no longer satisfied, condition (iii) does not hold. Under these conditions, the expression for X^* is then:

$$X^* \approx \frac{k_1 K_{m2}}{k_2 K_{m1}} \frac{X_T}{M_T} Z(1 - \frac{p_T}{X_T} c) \text{ and } X^*_{\text{is}} \approx \frac{k_1 K_{m2}}{k_2 K_{m1}} \frac{X_T}{M_T} Z_{\text{is}}. \tag{23}$$

From (21)-(23), we have $\underline{\Psi}(U, v)$ given by:

$$\underline{\psi} \approx \left[\begin{array}{ccc} \frac{X_T}{K_{m1}} Z(1 - \frac{p_T}{X_T} c), & \frac{k_1}{k_2} \frac{X_T}{K_{m1}} Z(1 - \frac{p_T}{X_T} c), & \frac{k_1 K_{m2}}{k_2 K_{m1}} \frac{X_T}{M_T} Z(1 - \frac{p_T}{X_T} c) \end{array} \right]^T_{3\times 1}. \tag{24}$$

Solving for ϕ by setting $s(\underline{X}, v) = 0$, we have:

$$\begin{array}{c} k_{\text{on}} X^* (1 - c) = k_{\text{off}} c, \\ \text{i.e., } X^* - X^* c = k_D c, \\ \text{i.e., } \phi = c = \dfrac{X^*}{k_D + X^*}. \end{array} \tag{25}$$

We can use (24) and (25) to find $\underline{\Gamma}$ as defined in Remark 1, and find that it satisfies Assumption 7. We then state without proof the following claims for this system:

Claim 1. *For the matrix B and functions r, f_1 and s defined in Table 1, Assumption 3 is satisfied for this system.*

Claim 2. *For the functions f_0 and $\underline{r}$ and matrices R, S_1 and A defined in Table 1, and the functions $\underline{\gamma}$ and ϕ as found above, Assumption 9 is satisfied for this system.*

For matrices T, Q, M, P defined in Table 1, we see that Assumption 4 is satisfied. Further, for $\underline{\Psi}$ and ϕ defined by (24) and (25), Assumption 5 and 6 are satisfied. Thus, Theorems 1, 2 and 3 can be applied to this system to check if the system can transmit unidirectional signals according to Definition 1 by varying X_T and M_T.

Results: (i) Retroactivity to the input: Using Theorem 1, we see that since $S_1 = 0$ from Table 1, $h_2 = 0$. Since $|R\underline{\Gamma}(U)| = \frac{X_T}{K_{m1}} Z$, to have small h_1, we must have a small $\frac{X_T}{K_{m1}}$. Evaluating the final term, we see that:

$$\left| \left(T^{-1} M \frac{\partial \underline{\Gamma}(U)}{\partial U} + T^{-1} M Q^{-1} P \frac{\partial \phi}{\partial \underline{X}}\bigg|_{\underline{X} = \underline{\Gamma}(U)} \frac{\partial \underline{\Gamma}(U)}{\partial U} \right) \dot{U} \right| = \frac{X_T}{K_{m1}} |\dot{Z}|.$$

Thus, for a small h_3, we must again have a small $\frac{X_T}{K_{m1}}$. Thus, for a small retroactivity to the input, we must have small $\frac{X_T}{K_{m1}}$.

(ii) Retroactivity to the output: Using Theorem 2, we see that since $S_1 = 0$, $h_2 = 0$. Further, the term $\left| \left(T^{-1} M Q^{-1} P \frac{\partial \phi}{\partial \underline{x}}\Big|_{\underline{x} = \underline{\gamma}(u)} \frac{\partial \underline{\gamma}(u)}{\partial u} \right) \dot{u} \right| = 0$ since $T^{-1} M Q^{-1} P = 0$ from Table 1. Thus, $\bar{h}_3 = 0$. For term $\bar{h}_1$ to be small, we see that $S_2 = S_3 = \frac{p_T}{X_T}$ must be small. Thus, to decrease the retroactivity to input, X_T must be increased.

(iii) Input-output relationship: Using Theorem 3, we know that $\underline{X}_{is} = \underline{\Gamma}_{is} + \mathcal{O}(\epsilon)$. Thus, $Y_{is} = I\underline{\Gamma}_{is} + \mathcal{O}(\epsilon)$. Under Remark 1, $I\underline{\Gamma}_{is} = I\underline{\Psi}(U_{is}, 0) \approx \frac{k_1 K_{m2}}{k_2 K_{m1}} \frac{X_T}{M_T} Z_{\text{is}}$ from (24). Thus, the dimensionless input-output behavior is approximately linear. Thus, from Def. 1(iii) we have that $m = 1$ and $K = \frac{k_1 K_{m2}}{k_2 K_{m1}} \frac{X_T}{M_T}$ which can be tuned by tuning the substrate and phosphatase concentrations X_T, M_T.

5.3 Double cycle with input as kinase of both phosphorylations

The reactions for this system are then:

$$Z \underset{k(t)}{\overset{\delta}{\rightleftharpoons}} \phi, \qquad X \underset{k_X}{\overset{\delta}{\rightleftharpoons}} \phi, \tag{26}$$

$$M \underset{k_M}{\overset{\delta}{\rightleftharpoons}} \phi, \qquad C_1, C_2, C_3, C_4, X^*, X^{**}, C \xrightarrow{\delta} \phi, \tag{27}$$

$$Z + X \underset{d_1}{\overset{a_1}{\rightleftharpoons}} C_1 \xrightarrow{k_1} X^* + Z, \qquad X^* + M \underset{d_2}{\overset{a_2}{\rightleftharpoons}} C_2 \xrightarrow{k_2} M + X, \tag{28}$$

$$X^* + Z \underset{d_3}{\overset{a_3}{\rightleftharpoons}} C_3 \xrightarrow{k_3} X^{**} + Z, \qquad X^{**} + M \underset{d_4}{\overset{a_4}{\rightleftharpoons}} C_4 \xrightarrow{k_4} X^* + M, \tag{29}$$

$$X^{**} + p \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} C. \tag{30}$$

Using the reaction-rate equations, the ODEs for this system are:

$$\begin{aligned}
\frac{dZ}{dt} &= k(t) - \delta Z - a_1 ZX + (d_1 + k_1)C_1 - a_3 X^* Z + (d_3 + k_3)C_3, && Z(0) = 0,\\
\frac{dX}{dt} &= k_X - \delta X - a_1 ZX + d_1 C_1 + k_2 C_2, && X(0) = \frac{k_X}{\delta},\\
\frac{dM}{dt} &= k_M - \delta M - a_2 X^* M + (d_2 + k_2)C_2 - a_4 X^{**} M + (d_4 + k_4)C_4, && M(0) = \frac{k_M}{\delta},\\
\frac{dC_1}{dt} &= a_1 ZX - (d_1 + k_1)C_1 - \delta C_1, && C_1(0) = 0,\\
\frac{dC_2}{dt} &= a_2 X^* M - (d_2 + k_2)C_2 - \delta C_2, && C_2(0) = 0,\\
\frac{dX^*}{dt} &= k_1 C_1 - a_2 X^* M - a_3 X^* Z + k_4 C_4 + d_2 C_2 + d_3 C_3 - \delta X^*, && X^*(0) = 0,\\
\frac{dC_3}{dt} &= a_3 X^* Z - (d_3 + k_3)C_3 - \delta C_3, && C_3(0) = 0,\\
\frac{dC_4}{dt} &= a_4 X^{**} M - (d_4 + k_4)C_4 - \delta C_4, && C_4(0) = 0,\\
\frac{dX^{**}}{dt} &= k_3 C_3 - a_4 X^{**} M + d_4 C_4 - \delta X^{**} - k_{\text{on}} X^{**}(p_{\text{T}} - C) + k_{\text{off}} C, && X^{**}(0) = 0,\\
\frac{dC}{dt} &= k_{\text{on}} X^{**}(p_{\text{T}} - C) - k_{\text{off}} C - \delta C, && C(0) = 0.
\end{aligned} \tag{31}$$

For system (31), let $M_T = M + C_2 + C_4$. Then its dynamics are $\dot{M}_T = k_M - \delta M_T$, $M_T(0) = \frac{k_M}{\delta}$. This gives a constant $M_T(t) = \frac{k_M}{\delta}$. The variable $M = M_T - C_2 - C_4$ can then be eliminated from the system. Similarly, defining $X_T = X + C_1 + C_2 + X^* + C_3 + C_4 + X^{**} + C$ gives a constant $X_T(t) = \frac{k_X}{\delta}$, and X can be eliminated from the system as $X = X_T - X^* - X^{**} - C_1 - C_2 - C_3 - C_4 - C$. Further, we define $c = \frac{C}{p_T}$ which the dimensionless form of C. The

U	Z	v	c
$\underline{x}$	$\begin{bmatrix} C_1 & C_2 & X^* & C_3 & C_4 & X^{**} \end{bmatrix}_{6\times1}^T$	$Y,\ I$	$X^{**}, \begin{bmatrix} 0 & 0 & 0 & 0 & 0 & 1 \end{bmatrix}_{1\times6}$
G_1	$\max\left\{\frac{a_1X_T}{\delta}, \frac{d_1}{\delta}, \frac{k_1}{\delta}, \frac{a_2M_T}{\delta}, \frac{d_2}{\delta}, \frac{k_2}{\delta}, \frac{a_3X_T}{\delta}, \frac{d_3}{\delta}, \frac{k_3}{\delta}, \frac{a_4M_T}{\delta}, \frac{d_4}{\delta}, \frac{k_4}{\delta}\right\}$	G_2	$\max\left\{\frac{k_{\text{on}}p_T}{\delta}, \frac{k_{\text{off}}}{\delta}\right\}$
$f_0(U, R\underline{X}, S_1v, t)$	$k(t) - \delta Z - \delta C_1 - \delta C_3$	$s(\underline{X}, v)$	$\frac{1}{G_2}\left(k_{\text{on}}X^{**}(1-c) - k_{\text{off}}c - \delta c\right)$
$\underline{r}(U, \underline{X}, S_2v)$	$\frac{1}{G_1}\begin{bmatrix} -a_1ZX_T(1 - \frac{X^*}{X_T} - \frac{X^{**}}{X_T} - \frac{C_1}{X_T} - \frac{C_2}{X_T} - \frac{C_3}{X_T} - \frac{C_4}{X_T} - \frac{p_T}{X_T}c) + (d_1+k_1)C_1 + \delta C_1 \\ -a_3ZX^* + (d_3+k_3)C_3 + \delta C_3 \end{bmatrix}_{2\times1}$		
$f_1(U, \underline{X}, S_3v)$	$\frac{1}{G_1}\begin{bmatrix} 0 \\ a_2X^*(M_T - C_2 - C_4) - (d_2+k_2)C_2 - \delta C_2 \\ k_1C_1 - a_2X^*(M_T - C_2 - C_4) - a_3X^*Z + k_4C_4 + d_2C_2 + d_3C_3 - \delta X^* \\ 0 \\ a_4X^{**}(M_T - C_2 - C_4) - (d_4+k_4)C_4 - \delta C_4 \\ k_3C_3 - a_4M_T(X^{**} + \frac{\delta p_T}{a_4M_T}c)a_4X^{**}(C_2+C_4) + d_4C_4 - \delta X^{**} \end{bmatrix}_{6\times1}$		
A	$\begin{bmatrix} 1 & 1 \end{bmatrix}_{1\times2}$	D	1
B	$\begin{bmatrix} -1 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & -1 & 0 & 0 \end{bmatrix}_{6\times2}^T$	C	$\begin{bmatrix} 0 & 0 & 0 & 0 & 0 & -p_T \end{bmatrix}_{6\times1}^T$
R	$\begin{bmatrix} 1 & 0 & 0 & 1 & 0 & 0 \end{bmatrix}_{1\times6}$	S_1	0
S_2	$\frac{p_T}{X_T}$	S_3	$\frac{\delta p_T}{a_4M_T}$
T	1	M	$\begin{bmatrix} 1 & 0 & 0 & 1 & 0 & 0 \end{bmatrix}_{1\times6}$
Q	$\mathbb{I}_{6\times6}$	P	$\begin{bmatrix} 0 & 0 & 0 & 0 & 0 & p_T \end{bmatrix}_{6\times1}^T$

Table 2. System variables, functions and matrices for a double phosphorylation cycle with the kinase for both cycles as input brought to form (1).

system then reduces to:

$$
\begin{aligned}
\frac{dZ}{dt} &= k(t) - \delta Z - a_1Z(X_T - X^* - X^{**} - C_1 - C_2 - C_3 - C_4 - p_Tc) + (d_1+k_1)C_1 - a_3X^*Z + (d_3+k_3)C_3, && Z(0) = 0,\\
\frac{dC_1}{dt} &= a_1Z(X_T - X^* - X^{**} - C_1 - C_2 - C_3 - C_4 - p_Tc) - (d_1+k_1)C_1 - \delta C_1, && C_1(0) = 0,\\
\frac{dC_2}{dt} &= a_2X^*(M_T - C_2 - C_4) - (d_2+k_2)C_2 - \delta C_2, && C_2(0) = 0,\\
\frac{dX^*}{dt} &= k_1C_1 - a_2X^*(M_T - C_2 - C_4) - a_3X^*Z + k_4C_4 + d_2C_2 + d_3C_3 - \delta X^*, && X^*(0) = 0,\\
\frac{dC_3}{dt} &= a_3X^*Z - (d_3+k_3)C_3 - \delta C_3, && C_3(0) = 0,\\
\frac{dC_4}{dt} &= a_4X^{**}(M_T - C_2 - C_4) - (d_4+k_4)C_4 - \delta C_4, && C_4(0) = 0,\\
\frac{dX^{**}}{dt} &= k_3C_3 - a_4X^{**}(M_T - C_2 - C_4) + d_4C_4 - \delta X^{**}\\
&\quad - k_{\text{on}}X^{**}p_T(1-c) + k_{\text{off}}p_Tc, && X^{**}(0) = 0,\\
\frac{dC}{dt} &= k_{\text{on}}X^{**}(1-c) - k_{\text{off}}c - \delta c, && c(0) = 0.
\end{aligned}
\tag{32}
$$

This system (32) is brought to form (1) as shown in Table 2.

For the system brought to form (1) as seen in Table 2, we now solve for $\underline{\Psi}$ and ϕ as defined by Assumptions 5 and 6.

Solving for $\underline{X} = \underline{\Psi}$ by setting $(Br + f_1)_{6\times 1} = 0$, we have:

$$
\begin{aligned}
&(Br + f_1)_2 = 0 \implies a_2 X_T^*(M_T - C_2 - C_4) = (d_2 + k_2 + \delta)\, C_2. \\
&\text{Under Assumption 1, } (d_2 + k_2) \gg \delta. \\
&\text{Then, } M_T X^* - X^* C_2 - X^* C_4 \approx K_{m2} C_2. \qquad (33)\\
&(Br + f_1)_5 = 0 \implies a_4 X^{**}(M_T - C_2 - C_4) = (d_4 + k_4 + \delta)\, C_4 \\
&\text{Under Assumption 1, } d_4 + k_4 \gg \delta. \\
&\text{Then, } M_T X^{**} - X^{**} C_2 - X^{**} C_4 \approx K_{m4} C_4. \qquad (34)\\
&\text{For } K_{m2} \gg X^* \text{ and } K_{m4} \gg X^{**}, \\
&C_2 \approx \frac{X^* M_T}{K_{m2}} \text{ and } C_4 \approx \frac{X^{**} M_T}{K_{m4}}.
\end{aligned}
$$

$$
\begin{aligned}
&(Br + f_1)_5 = 0 \text{ and } (Br + f_1)_6 = 0 \implies k_3 C_3 \approx k_4 C_4, \\
&\text{i.e., } C_3 \approx \frac{k_4}{k_3}\frac{X^{**} M_T}{K_{m4}}. \qquad (35)\\
&(Br + f_1)_3 = 0 \text{ and } (Br + f_1)_4 = 0 \implies k_1 C_1 \approx k_2 C_2, \\
&\text{i.e., } C_1 \approx \frac{k_2}{k_1}\frac{M_T X^*}{K_{m2}}. \qquad (36)
\end{aligned}
$$

634

$$
\begin{aligned}
&(Br + f_1)_4 = 0 \implies a_3 X^* Z = (d_3 + k_3) C_3, \\
&\text{i.e., from (35), } \frac{Z X^*}{K_{m3}} = C_3 \approx \frac{k_4}{k_3}\frac{X^{**} M_T}{K_{m4}}, \qquad (37)\\
&\text{i.e., } X^* \approx \frac{k_4 K_{m3}}{k_3 K_{m4}}\frac{X^{**} M_T}{Z}.
\end{aligned}
$$

635

$$
\begin{aligned}
&(Br + f_1)_1 = 0 \implies \\
&a_1 Z X_T (1 - \frac{X^*}{X_T} - \frac{X^{**}}{X_T} - \frac{C_1}{X_T} - \frac{C_2}{X_T} - \frac{C_3}{X_T} - \frac{C_4}{X_T} - \frac{p_T}{X_T} c) = (d_1 + k_1) C_1, \\
&\text{i.e., } Z\left(1 - \frac{k_4 K_{m3}}{k_3 K_{m4}}\frac{X^{**} M_T}{Z X_T} - \frac{X^{**}}{X_T} - (\frac{k_2}{k_1} + 1)\frac{M_T}{X_T K_{m2}}\frac{k_4 K_{m3}}{k_3 K_{m4}}\frac{X^{**} M_T}{Z}\right. \\
&\left. -(\frac{k_4}{k_3} + 1)\frac{X^{**} M_T}{X_T K_{m4}} - \frac{p_T}{X_T} c\right) \approx K_{m1}\frac{k_2}{k_1}\frac{M_T}{K_{m2}}\frac{k_4 K_{m3}}{k_3 K_{m4}}\frac{X^{**} M_T}{Z}. \\
&\text{i.e., } Z X_T (1 - \frac{p_T}{X_T} c) \qquad (38)\\
&\approx X^{**} + X^{**}\left(\frac{k_4 K_{m3}}{k_3 K_{m4}}\frac{M_T}{Z}\right)\left(\frac{M_T}{K_{m2}}(\frac{k_2}{k_1} + 1) + M_T\frac{k_2 K_{m1}}{k_1 K_{m2}} + \frac{k_3 Z}{k_4 K_{m3}}(\frac{k_4}{k_3} + 1)\right). \\
&\text{If } K_{m1}, K_{m2}, K_{m3}, K_{m4} \gg Z \text{ and } \frac{M_T}{Z} \gg 1, \\
&Z\left(1 - \frac{p_T}{X_T} c\right) \approx X^{**}\left(\frac{k_4 K_{m3}}{k_3 K_{m4}}\frac{M_T}{X_T}\frac{k_2 K_{m1}}{k_1 K_{m2}}\frac{M_T}{\bar{k} z}\right), \\
&\text{i.e., } X^{**} \approx \frac{X_T}{M_T^2} Z^2 \frac{k_3 K_{m4}}{k_4 K_{m3}}\frac{k_1 K_{m2}}{k_2 K_{m1}}\left(1 - \frac{p_T}{X_T} c\right).
\end{aligned}
$$

Thus, from (34)-(38), we have the function $\underline{\Psi}(U,v)$:

$$\underline{\Psi} \approx \begin{bmatrix} \left(\frac{ZX_T}{K_{m1}}\right)\left(1-\frac{p_T}{X_T}c\right), \\ \frac{k_1}{k_2}\left(\frac{ZX_T}{K_{m1}}\right)\left(1-\frac{p_T}{X_T}c\right), \\ \frac{k_1K_{m2}}{k_2K_{m1}}\left(\frac{ZX_T}{M_T}\right)\left(1-\frac{p_T}{X_T}c\right), \\ \frac{Z^2X_T}{M_T}\frac{1}{K_{m3}}\frac{k_1K_{m2}}{k_2K_{m1}}\left(1-\frac{p_T}{X_T}c\right), \\ \frac{Z^2X_T}{M_T}\frac{k_3}{k_4K_{m3}}\frac{k_1K_{m2}}{k_2K_{m1}}\left(1-\frac{p_T}{X_T}c\right), \\ \left(\frac{Z}{M_T}\right)^2 X_T\frac{k_3K_{m4}}{k_4K_{m3}}\frac{k_1K_{m2}}{k_2K_{m1}}\left(1-\frac{p_T}{X_T}c\right) \end{bmatrix}_{6\times 1}. \tag{39}$$

Solving for ϕ by setting $s(\underline{X},v)=0$, we have:

$$\begin{aligned} &k_{\text{on}}X^{**}(1-c) = k_{\text{off}}c, \\ &\text{i.e., } X^{**} - X^{**}c = k_D c, \\ &\text{i.e., } \phi = c = \frac{X^{**}}{k_D + X^{**}}. \end{aligned} \tag{40}$$

We can use (39) and (40) to find $\underline{\Gamma}$ as defined in Remark 1, and find that it satisfies Assumption 7. We then state the following claims without proof for this system:

Claim 3. *For the matrix B and the functions r, f_1 and s defined in Table 2, Assumption 3 is satisfied for large $K_{m1}, K_{m2}, K_{m3}, K_{m4}$.*

Claim 4. *For the functions f_0 and $\underline{r}$ and matrices R, S_1 and A defined in Table 2, and the functions $\underline{\gamma}$ and ϕ as found above, Assumption 9 is satisfied for this system.*

For matrices T, Q, M, P defined in Table 2, we see that Assumption 4 is satisfied. Further, for $\underline{\Psi}$ and ϕ defined by (39) and (40), Assumptions 5 and 6 are satisfied. Thus, Theorems 1, 2 and 3 can be applied to this system.

Results: (i) Retroactivity to the input: Using Theorem 1, we see that since $S_1 = 0$ from Table 2, $h_2 = 0$. Further, $R|\underline{\Gamma}(U)| = Z\frac{X_T}{K_{m1}} + Z^2\frac{X_T}{M_TK_{m3}}\frac{k_1K_{m2}}{k_2K_{m1}}$. For the final term h_3, we evaluate:

$$\left|\left(T^{-1}M\frac{\partial\underline{\Gamma}(U)}{\partial U} + T^{-1}MQ^{-1}P\frac{\partial\phi}{\partial\underline{X}}\bigg|_{\underline{X}=\underline{\Gamma}(U)}\frac{\partial\underline{\Gamma}(U)}{\partial U}\right)\dot{U}\right| = \left(\frac{X_T}{K_{m1}} + 2Z\frac{X_T}{M_TK_{m3}}\frac{k_1K_{m2}}{k_2K_{m1}}\right)\dot{Z}.$$

Thus, for small h_1 and h_3, and therefore small retroactivity to the input, we must have small $\frac{X_T}{K_{m1}}$ and $\frac{X_T}{M_TK_{m3}}\frac{k_1K_{m2}}{k_2K_{m1}}$.

(ii) Retroactivity to the output: From Table 2, we see that $S_1 = 0$. Thus, $h_2 = 0$. Further, evaluating the expression $\left|\left(T^{-1}MQ^{-1}P\frac{\partial\phi(\underline{X})}{\partial\underline{X}}\bigg|_{\underline{X}=\underline{\Gamma}(U)}\frac{\partial\underline{\Gamma}(U)}{\partial U}\right)\dot{U}\right|$ gives $\bar{h}_3 = 0$, since $T^{-1}MQ^{-1}P = 0$. For a small retroactivity to the output, then, we must have small $\bar{h}_1$. Since $S_3 = 0$, we must have a small $S_2 = \frac{p_T}{X_T}$. Thus, for a small retroactivity to the output, we must have a large X_T.

(iii) Input-output relationship: From eqn. (39), we have that:

$$Y_{is} = I\underline{X}_{is} \approx I\underline{\Gamma}_{is} = I\underline{\Psi}(U_{is},0) \approx \frac{X_T}{M_T^2}Z_{is}^2\frac{k_3K_{m4}}{k_4K_{m3}}\frac{k_1K_{m2}}{k_2K_{m1}}. \tag{41}$$

5.4 Phosphotransfer with kinase as input

The reactions for this system are:

$$Z \underset{k(t)}{\overset{\delta}{\rightleftharpoons}} \phi, \qquad X_1 \underset{k_{X_1}}{\overset{\delta}{\rightleftharpoons}} \phi, \tag{42}$$

$$X_2 \underset{k_{X_2}}{\overset{\delta}{\rightleftharpoons}} \phi, \qquad M \underset{k_M}{\overset{\delta}{\rightleftharpoons}} \phi, \tag{43}$$

$$C_1, X_1^*, X_2^*, C_2, C_4, C \xrightarrow{\delta} \phi, \qquad X_1 + Z \underset{d_1}{\overset{a_1}{\rightleftharpoons}} C_1 \xrightarrow{k_1} X_1^* + Z, \tag{44}$$

$$X_1^* + X_2 \underset{d_2}{\overset{a_2}{\rightleftharpoons}} C_2 \underset{a_3}{\overset{d_3}{\rightleftharpoons}} X_1 + X_2^*, \qquad X_2^* + M \underset{d_4}{\overset{a_4}{\rightleftharpoons}} C_4 \xrightarrow{k_4} X_2 + M, \tag{45}$$

$$X_2^* + p \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} C. \tag{46}$$

The ODEs based on the reaction rate equations are:

$$\begin{aligned}
\dot{Z} &= k(t) - \delta Z - a_1 X_1 Z + (d_1 + k_1) C_1, & Z(0) &= 0,\\
\dot{X}_1 &= k_{X_1} - \delta X_1 - a_1 X_1 Z + d_1 C_1 + d_3 C_2 - a_3 X_1 X_2^*, & X_1(0) &= \frac{k_{X_1}}{\delta},\\
\dot{C}_1 &= a_1 X_1 Z - (d_1 + k_1) C_1 - \delta C_1, & C_1(0) &= 0,\\
\dot{X}_1^* &= k_1 C_1 - a_2 X_1^* X_2 + d_2 C_2 - \delta X_1^*, & X_1^*(0) &= 0,\\
\dot{X}_2 &= k_{X_2} - \delta X_2 - a_2 X_1^* X_2 + d_2 C_2 + k_4 C_4, & X_2(0) &= \frac{k_{X_2}}{\delta},\\
\dot{C}_2 &= a_2 X_1^* X_2 + a_3 X_1 X_2^* - (d_2 + d_3) C_2 - \delta C_2, & C_2(0) &= 0,\\
\dot{X}_2^* &= d_3 C_2 - a_3 X_1 X_2^* - a_4 X_2^* M + d_4 C_4 - \delta X_2^* - k_{\text{on}} X_2^* (p_T - C) + k_{\text{off}} C, & X_2^*(0) &= 0,\\
\dot{C}_4 &= a_4 X_2^* M - (d_4 + k_4) C_4 - \delta C_4, & C_4(0) &= 0,\\
\dot{M} &= k_M - \delta M - a_4 X_2^* M + (d_4 + k_4) C_4, & M(0) &= \frac{k_M}{\delta},\\
\dot{C} &= k_{\text{on}} X_2^* (p_T - C) - k_{\text{off}} C - \delta C, & C(0) &= 0.
\end{aligned} \tag{47}$$

For (47), define $X_{T1} = X_1 + C_1 + X_1^* + C_2$. Then, $\dot{X}_{T1} = k_{X_1} - \delta X_{T1}, X_{T1}(0) = \frac{k_{X_1}}{\delta}$. Thus, $X_{T1}(t) = \frac{k_{X_1}}{\delta}$ is a constant at all time $t > 0$. Similarly, $X_{T2} = X_2 + C_2 + X_2^* + C_3 + C$ is a constant with $X_{T2}(t) = \frac{k_{X_2}}{\delta}$ and $M_T = M + C_3$ is a constant with $M_T(t) = \frac{k_M}{\delta}$ for all time $t > 0$. Thus, the variables $X_1 = X_{T1} - C_1 - X_1^* - C_2$, $X_2 = X_{T2} - C_2 - X_2^* - C_3 - C$ and $M = M_T - C_4$ can be eliminated from the system. Further, we define $c = \frac{C}{p_T}$. The reduced system is then:

$$\begin{aligned}
\dot{Z} &= k(t) - \delta Z - a_1 Z(X_{T1} - C_1 - X_1^* - C_2) + (d_1 + k_1) C_1, & Z(0) &= 0,\\
\dot{C}_1 &= a_1 Z(X_{T1} - C_1 - X_1^* - C_2) - (d_1 + k_1) C_1 - \delta C_1, & C_1(0) &= 0,\\
\dot{X}_1^* &= k_1 C_1 - a_2 X_1^* (X_{T2} - C_2 - X_2^* - C_4 - p_T c) + d_2 C_2 - \delta X_1^*, & X_1^*(0) &= 0,\\
\dot{C}_2 &= a_2 X_1^* (X_{T2} - C_2 - X_2^* - C_4 - p_T c) + a_3 (X_{T1} - C_1 - X_1^* - C_2) X_2^* - (d_2 + d_3) C_2 - \delta C_2, & C_2(0) &= 0,\\
\dot{X}_2^* &= d_3 C_2 - a_3 (X_{T1} - C_1 - X_1^* - C_2) X_2^* - a_4 X_2^* (M_T - C_4) + d_4 C_4 - \delta X_2^* - k_{\text{on}} X_2^* p_T (1 - c) + k_{\text{off}} p_T c, & X_2^*(0) &= 0,\\
\dot{C}_4 &= a_4 X_2^* (M_T - C_4) - (d_4 + k_4) C_4 - \delta C_4, & C_4(0) &= 0,\\
\dot{c} &= k_{\text{on}} X_2^* (1 - c) - k_{\text{off}} c - \delta c, & c(0) &= 0.
\end{aligned} \tag{48}$$

This system (48) is brought to form (1) as shown in Table 3.

We now solve for the functions $\underline{\Psi}$ and ϕ as defined by Assumptions 5 and 6.

U	Z	v	c
$\underline{X}$	$\begin{bmatrix} C_1 & X_1^* & C_2 & X_2^* & C_4 \end{bmatrix}^T_{5\times1}$	$Y,\ I$	$X_2^*, \begin{bmatrix} 0 & 0 & 0 & 1 & 0 \end{bmatrix}_{1\times5}$
G_1	$\max\left\{\frac{a_1X_{T1}}{\delta}, \frac{d_1}{\delta}, \frac{k_1}{\delta}, \frac{a_2X_{T2}}{\delta}, \frac{d_2}{\delta}, \frac{d_3}{\delta}, \frac{a_3X_{T1}}{\delta}, \frac{a_4M_T}{\delta}, \frac{d_4}{\delta}, \frac{k_4}{\delta}\right\}$	G_2	$\max\left\{\frac{k_{\text{on}}p_T}{\delta}, \frac{k_{\text{off}}}{\delta}\right\}$
$f_0(U, R\underline{X}, S_1v, t)$	$k(t) - \delta Z - \delta C_1$	$s(\underline{X}, v)$	$\frac{1}{G_2}\left(k_{\text{on}}X_2^*(1-c) - k_{\text{off}}c - \delta c\right)$
$\underline{r}(U, \underline{X}, S_2v)$	$\frac{1}{G_1}\left(-a_1Z(X_{T1} - C_1 - X_1^* - C_2) + (d_1+k_1)C_1 + \delta C_1\right)$		
$f_1(U, \underline{X}, S_3v)$	$\frac{1}{G_1}\begin{bmatrix} 0 \\ k_1C_1 - a_2X_1^*X_{T2}(1 - \frac{C_2}{X_{T2}} - \frac{X_2^*}{X_{T2}} - \frac{C_4}{X_{T2}} - \frac{p_T}{X_{T2}}c) + d_2C_2 - \delta X_1^*, \\ a_2X_1^*X_{T2}(1 - \frac{C_2}{X_{T2}} - \frac{X_2^*}{X_{T2}} - \frac{C_4}{X_{T2}} - \frac{p_T}{X_{T2}}c) - (d_2+d_3)C_2 + a_3(X_{T1} - C_1 - X_1^* - C_2)X_2^* - \delta C_2, \\ d_3C_2 - a_4X_2^*(M_T - C_4) + d_4C_4 + a_3(C_1 + X_1^* + C_2)X_2^* - a_3X_{T1}(X_2^* + \frac{\delta p_T}{a_3X_{T1}}c) - \delta X_2^*, \\ a_4X_2^*(M_T - C_4) - (d_4+k_4)C_4 - \delta C_4 \end{bmatrix}_{5\times1}$		
A	1	D	1
B	$\begin{bmatrix} -1 & 0 & 0 & 0 & 0 \end{bmatrix}^T_{5\times1}$	C	$\begin{bmatrix} 0 & 0 & 0 & -p_T & 0 \end{bmatrix}^T_{5\times1}$
R	$\begin{bmatrix} 1 & 0 & 0 & 0 & 0 \end{bmatrix}_{1\times5}$	S_1	0
S_2	0	S_3	$\frac{p_T}{X_{T2}}, \frac{\delta p_T}{a_3X_{T1}}$
T	1	M	$\begin{bmatrix} 1 & 0 & 0 & 0 & 0 \end{bmatrix}_{1\times5}$
Q	$\mathbb{I}_{5\times5}$	P	$\begin{bmatrix} 0 & 0 & 0 & p_T & 0 \end{bmatrix}^T_{5\times1}$

Table 3. System variables, functions and matrices for a phosphotransfer system with kinase as input brought to form (1).

Solving for $\underline{X} = \underline{\Psi}$ by setting $(Br + f_1)_5 = 0$, we have:

$$(Br + f_1)_1 = 0 \implies ZX_{T1} - ZX_1^* - ZC_2 \approx (K_{m1} + Z)C_1, \text{ under Assumption 1.}$$

$$\text{If } K_{m1} \gg Z, \quad ZX_{T1} \approx K_{m1}C_1, \text{ i.e., } C_1 \approx \frac{ZX_{T1}}{K_{m1}}.$$

$$(Br + f_1)_2 + (Br + f_1)_3 + (Br + f_1)_4 + (Br + f_1)_5 = 0 \implies k_1C_1 - k_4C_4 \approx 0,$$

$$\text{i.e., } C_4 \approx \frac{k_1}{k_4}\frac{ZX_{T1}}{K_{m1}}.$$

$$(Br + f_1)_5 = 0 \implies X_2^*M_T \approx (X_2^* + K_{m4})C_4.$$

$$\text{If } K_{m4} \gg X_2^*, \quad X_2^* \approx \frac{K_{m4}}{M_T}\frac{k_1}{k_4}\frac{ZX_{T1}}{K_{m1}}.$$

$$(Br + f_1)_3 = 0 \implies$$

$$a_2X_1^*X_{T2}(1 - \frac{C_2}{X_{T2}} - \frac{X_2^*}{X_{T2}} - \frac{C_4}{X_{T2}} - \frac{p_T}{X_{T2}}c)$$

$$- (d_2+d_3)C_2 + a_3(X_{T1} - C_1 - X_1^* - C_2)X_2^* \approx 0.$$

$$\text{If } (d_2+d_3) \gg a_2X_1^* \text{ and } a_3X_{T1}, \ C_2 \approx \frac{a_2X_1^*X_{T2} + a_3X_2^*X_{T1}}{d_2 + a_3}.$$

$$(Br + f_1)_2 = 0$$

$$\implies k_1C_1 - a_2X_{T2}X_1^*(1 - \frac{C_2}{X_{T2}} - \frac{X_2^*}{X_{T2}} - \frac{C_4}{X_{T2}} - \frac{p_T}{X_{T2}}c) + d_2C_2 - \delta X_1^* = 0.$$

$$\text{If } d_2 \gg a_2X_1^*, \ d_2C_2 \approx a_2X_1^* - k_1c_1.$$

Solving the above 2 simultaneously, we obtain:

$$X_1^* \approx \frac{k_1 X_{T1}}{a_2 d_3 X_{T2} K_{m1}} \left(\frac{d_2 a_3 K_{m4} X_{T1}}{k_4 M_T} + d_2 + d_3\right) Z$$
$$\text{and } C_2 \approx \frac{a_3 X_{T2}}{d_2 + d_3} \left(\frac{d_2}{d_3} + \frac{X_{T1}}{X_{T2}}\right) \frac{k_1 K_{m4}}{k_4 K_{m1}} \frac{X_{T1}}{M_T} Z.$$

Thus, we have the function $\underline{\Psi}(U, v)$:

$$\underline{\Psi} \approx \begin{bmatrix} \frac{Z X_{T1}}{K_{m1}}, \\ \frac{k_1 X_{T1}}{a_2 d_3 X_{T2} K_{m1}} \left(\frac{d_2 a_3 K_{m4} X_{T1}}{k_4 M_T} + d_2 + d_3\right) Z, \\ \frac{a_3 X_{T2}}{d_2 + d_3} \left(\frac{d_2}{d_3} + \frac{X_{T1}}{X_{T2}}\right) \frac{k_1 K_{m4}}{k_4 K_{m1}} \frac{X_{T1}}{M_T} Z, \\ \frac{k_1 K_{m3}}{k_3 K_{m1}} \frac{X_{T1}}{M_T} Z, \\ \frac{k_1 X_{T1}}{k_4} \frac{Z}{K_{m1}} \end{bmatrix}^T_{5\times 1}. \tag{49}$$

Solving for ϕ by setting $s(\underline{X}, v) = 0$, we have:

$$\begin{aligned} &k_{\text{on}} X_2^* (1 - c) - k_{\text{off}} c - \delta c = 0. \\ &\text{Under Assumption 1, } X_2^* - X_2^* c \approx k_D c, \\ &\text{i.e., } \phi = c \approx \frac{X_2^*}{X_2^* + k_D}. \end{aligned} \tag{50}$$

Finding $\underline{\Gamma}$ from (49) and (50) under Remark 1, we see that it satisfies Assumption 7. For matrices T, Q, M and P as seen in Table 3, we see that Assumption 4 is satisfied. Functions f_0 and $\underline{r}$ in Table 3 satisfy Assumptions 8. For the functions $\underline{\Psi}$, ϕ and $\underline{\Gamma}$, Assumptions 5, 6 and 7 are satisfied. We also claim without proof that Assumptions 3 and 9 are satisfied for this system. Theorems 1, 2 and 3 can then be applied to this system.

Results: (i) Retroactivity to the input: Using Theorem 1, since $S_1 = 0$ from Table 3, $h_2 = 0$. Further, $|R\underline{\Gamma}(U)| = \frac{X_{T1}}{K_{m1}} Z$. Finally, we evaluate the following expression for h_3:

$$\left| \left(T^{-1} M \frac{\partial \underline{\Gamma}(U)}{\partial U} + T^{-1} M Q^{-1} P \frac{\partial \phi}{\partial \underline{X}}\bigg|_{\underline{X} = \underline{\Gamma}(U)} \frac{\partial \underline{\Gamma}(U)}{\partial U} \right) \dot{U} \right| \approx \frac{X_{T1}}{K_{m1}} \dot{Z}.$$

Thus, for small h_1 and h_3, and therefore small retroactivity to the input, we must have small $\frac{X_{T1}}{K_{m1}}$.

(ii) Retroactivity to the output: Using Claim 2, we see from Table 3 that $S_1 = 0$, thus, $h_2 = 0$. Further, since $T^{-1} M Q^{-1} P = 0$, we find $\bar{h}_3 = 0$. For a small retroactivity to the output then, we must have a small $\bar{h}_1$. Since $S_2 = 0$, we must have a small $S_3 = \frac{p_T}{X_{T2}}, \frac{\delta p_T}{a_3 X_{T1}}$. Thus, for a small retroactivity to the output, we must have a large X_{T2} and $\frac{X_{T1} d_3}{\delta}$ compared to p_T.

(iii) Input-output relationship: From (49), we see that

$$X_{2,is}^* = I \underline{X}_{is} \approx I \underline{\Gamma}_{is} = I \underline{\Psi}(U_{is}, 0) \approx \frac{k_1 K_{m3}}{k_3 K_{m1}} \frac{X_{T1}}{M_T} Z_{is}. \tag{51}$$

5.5 N-stage cascade of single phosphorylation cycles with common phosphatase

The two-step reactions for the cascade are shown below. The reactions involving species of the first cycle are given by:

$$\phi \underset{\delta}{\overset{k(t)}{\rightleftharpoons}} Z, \qquad X_1 + Z \underset{d_{11}}{\overset{a_{11}}{\rightleftharpoons}} C_{11} \xrightarrow{k_{11}} X_1^* + Z, \tag{52}$$

$$X_1^* + M \underset{\beta_{21}}{\overset{\beta_{11}}{\rightleftharpoons}} C_{21} \xrightarrow{k_{21}} X_1 + M, \tag{53}$$

$$X_1^* + X_2 \underset{d_{12}}{\overset{a_{12}}{\rightleftharpoons}} C_{12} \xrightarrow{k_{12}} X_1^* + X_2^*. \tag{54}$$

The reactions involving species of the i^{th} cycle, for $i \in [2, N-1]$, are given by:

$$X_i + X_{i-1}^* \underset{d_{1i}}{\overset{a_{1i}}{\rightleftharpoons}} C_{1i} \xrightarrow{k_{1i}} X_i^* + X_{i-1}^*,\ K_{m1i} = \frac{d_{1i} + k_{1i}}{a_{1i}}, \tag{55}$$

$$X_i^* + M \underset{\beta_{2i}}{\overset{\beta_{1i}}{\rightleftharpoons}} C_{2i} \xrightarrow{k_{2i}} X_i + M,\ K_{m2i} = \frac{\beta_{2i} + k_{2i}}{\beta_{1i}}, \tag{56}$$

$$X_i^* + X_{i+1} \underset{d_{1_{i+1}}}{\overset{a_{1_{i+1}}}{\rightleftharpoons}} C_{1_{i+1}} \xrightarrow{k_{1_{i+1}}} X_i^* + X_{i+1}^*. \tag{57}$$

And those for the final cycle are given by:

$$X_N + X_{N-1}^* \underset{d_{1N}}{\overset{a_{1N}}{\rightleftharpoons}} C_{1N} \xrightarrow{k_{1N}} X_N^* + X_{N-1}^*, \tag{58}$$

$$X_N^* + M \underset{\beta_{2N}}{\overset{\beta_{1N}}{\rightleftharpoons}} C_{2N} \xrightarrow{k_{2N}} X_N + M, \tag{59}$$

$$X_N^* + p \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} C. \tag{60}$$

The production and dilution of the proteins and other species gives:

$$X_i \underset{k_{Xi}}{\overset{\delta}{\rightleftharpoons}} \phi, \quad M \underset{k_M}{\overset{\delta}{\rightleftharpoons}} \phi, \quad C_{1i}, X_i^*, C_{2i}, C \xrightarrow{\delta} \phi.$$

The reaction rate equations for the system are then given below, for time $t \in [t_i, t_f]$. For the input,

$$\dot{Z} = k(t) - \delta Z - a_{11} X_1 Z + (d_{11} + k_{11}) C_{11}. \tag{61}$$

For the first cycle,

$$\dot{X}_1 = k_{X1} - \delta X_1 - a_{11} X_1 Z + d_{11} C_{11} + k_{21} C_{21}, \qquad X_1(0) = \frac{k_{X1}}{\delta}, \tag{62}$$

$$\dot{C}_{11} = a_{11} X_1 Z - (d_{11} + k_{11}) C_{11} - \delta C_{11}, \qquad C_{11}(0) = 0, \tag{63}$$

$$\dot{C}_{21} = \beta_{11} X_1^* M - (\beta_{21} + k_{21}) C_{21} - \delta C_{21}, \qquad C_{21}(0) = 0, \tag{64}$$

$$\dot{X}_1^* = k_{11} C_{11} - \beta_{11} X_1^* M + \beta_{21} C_{21} - a_{12} X_1^* X_2 \tag{65}$$

$$+ (d_{12} + k_{12}) C_{12} - \delta X_1^*, \qquad X_1^*(0) = 0. \tag{66}$$

For the i^{th} cycle, where $i \in [2, N-1]$:

$$\dot{X}_i = k_{Xi} - \delta X_i - a_{1i} X_i X_{i-1}^* + d_{1i} C_{1i} + k_{2i} C_{2i}, \qquad X_i(0) = \frac{k_{Xi}}{\delta}, \tag{67}$$

$$\dot{C}_{1i} = a_{1i} X_i X_{i-1}^* - (d_{1i} + k_{1i}) C_{1i} - \delta C_{1i}, \qquad C_{1i}(0) = 0, \tag{68}$$

$$\dot{C}_{2i} = \beta_{1i} X_i^* M - (\beta_{2i} + k_{2i}) C_{2i} - \delta C_{2i}, \qquad C_{2i}(0) = 0, \tag{69}$$

$$\dot{X}_i^* = k_{1i} C_{1i} - \beta_{1i} X_i^* M + \beta_{2i} C_{2i} - a_{1_{i+1}} X_i^* X_{i+1} \tag{70}$$

$$+ (d_{1_{i+1}} + k_{1_{i+1}}) C_{1_{i+1}} - \delta X_i^*, \qquad X_i^*(0) = 0. \tag{71}$$

For the last, N^{th}, cycle:

$$\dot{X}_N = k_{XN} - \delta X_N - a_{1N} X_N X_{N-1}^* + d_{1N} C_{1N} + k_{2N} C_{2N}, \qquad X_N(0) = \frac{k_{XN}}{\delta}, \tag{72}$$

$$\dot{C}_{1N} = a_{1N} X_N X_{N-1}^* - (d_{1N} + k_{1N}) C_{1N} - \delta C_{1N}, C_{1N}(0) = 0, \qquad C_{1N}(0) = 0, \tag{73}$$

$$\dot{C}_{2N} = \beta_{1N} X_N^* M - (\beta_{2N} + k_{2N}) C_{2N} - \delta C_{2N}, \qquad C_{2N}(0) = 0, \tag{74}$$

$$\dot{X}_N^* = k_{1N} C_{1N} - \beta_{1N} X_N^* M + \beta_{2N} C_{2N} \tag{75}$$

$$- k_{\text{on}}(p_T - C) X_N^* + k_{\text{off}} C - \delta X_N^*, \qquad X_N^*(0) = 0. \tag{76}$$

U	Z	v	c
$\underline{x}$	$\left[\begin{array}{ccccccc} C_{11} & ... & C_{1i} & C_{2i} & X_i^* & ... & X_N^* \end{array}\right]^T_{3N\times 1}$	$Y,\ I$	$X_N^*, \left[\begin{array}{cccccc} 0 & 0 & ... & 0 & 1 \end{array}\right]_{1\times 3N}$
G_1	$G_1 = \min\left\{\frac{a_1 X_{Ti}}{\delta}, \frac{d_1}{\delta}, \frac{k_1}{\delta}, \frac{a_2 M_T}{\delta}, \frac{d_2}{\delta}, \frac{k_2}{\delta}\right\}$	G_2	$\min\left\{\frac{k_{\text{on}} p_T}{\delta}, \frac{k_{\text{off}}}{\delta}\right\}$
$f_0(U, R\underline{X}, S_1 v, t)$	$k(t) - \delta Z - \delta C_{11}$	$s(\underline{X}, v)$	$\frac{1}{G_2}\left(k_{\text{on}} X_N^*(1-c) - k_{\text{off}} c - \delta c\right)$
$\underline{r}(u, \underline{x}, S_2 v)$	$\frac{1}{G_1}\left[\begin{array}{cc} -a_1 Z(X_{T1} - C_{11} - X_1^* - C_{21} - C_{12}) + (d_1+k_1)C_{11} + \delta C_{11} \end{array}\right]_{1\times 1}$		
$f_1(u, \underline{x}, S_3 v)$	$\frac{1}{G_1}\left[\begin{array}{c} 0 \\ a_2(M_T - \sum C_{2i})X_1^* - (d_2+k_2)C_{21} - \delta C_{2i}, \\ k_1 C_{11} - a_2 X_1^*(M_T - \sum C_{2i}) + d_2 C_{21} - a_1 X_1^*(X_{T2} - C_{12} - X_2^* - C_{22} - C_{13}) + (d_1+k_1)C_{12} - \delta X_1^* \\ ... \\ a_1 X_{i-1}^*(X_{Ti} - C_{1i} - X_i^* - C_{2i} - C_{1_{i+1}}) - (d_1+k_1)C_{1i} - \delta C_{1i} \\ a_2(M_T - \sum C_{2i})X_i^* - (d_2+k_2)C_{2i} - \delta C_{2i} \\ k_1 C_{1i} - a_2 X_i^*(M_T - \sum C_{2i}) - a_1 X_i^*(X_{T_{i+1}} - C_{1_{i+1}} - X_{i+1}^* - C_{2_{i+1}} - C_{1_{i+2}}) + (d_1+k_1)C_{1_{i+1}} - \delta X_i^* \\ ... \\ a_1 X_{TN} X_{N-1}^*(1 - \frac{p_T}{X_{TN}} c) - a_1 X_{N-1}^*(C_{1N} + X_N^* + C_{2N}) - (d_1+k_1)C_{1N} - \delta C_{1N} \\ a_2 X_N^*(M_T - \sum C_{2i}) - (d_2+k_2)C_{2N} - \delta C_{2N} \\ k_1 C_{1N} - a_2 M_T(X_N^* + \frac{\delta p_T}{a_2 M_T} c) + a_2 X_N^* \sum C_{2i} + d_2 C_{2N} - \delta X_N^* \end{array}\right]_{3N\times 1}$		
A	1	D	1
B	$\left[\begin{array}{ccccc} -1 & 0 & ... & 0 \end{array}\right]^T_{3N\times 1}$	C	$\left[\begin{array}{ccccc} 0 & 0 & ... & 0 & -p_T \end{array}\right]^T_{3N\times 1}$
R	$\left[\begin{array}{cccc} 1 & 0 & ... & 0 \end{array}\right]_{1\times 3N}$	S_1	0
S_2	0	S_3	$\frac{p_T}{X_{TN}}, \frac{\delta p_T}{a_2 M_T}$
T	1	M	$\left[\begin{array}{cccc} 1 & 0 & ... & 0 \end{array}\right]_{1\times 3N}$
Q	$\mathbb{I}_{3N\times 3N}$	P	$\left[\begin{array}{ccccc} 0 & ... & 0 & p_T \end{array}\right]^T_{3N\times 1}$

Table 4. System variables, functions and matrices for an N-stage cascade of phosphorylation cycles with the kinase as input to the first cycle brought to form (1).

For the common phosphatase:

$$\dot{M} = k_M - \delta M - \sum_{i=1}^{i=N} (\beta_{1i} X_i^* M - (\beta_{2i} + k_{2i}) C_{2i}). \tag{77}$$

For the downstream system,

$$\dot{C} = k_{\text{on}}(p_T - C) X_N^* - k_{\text{off}} C - \delta C. \tag{78}$$

Seeing that $X_{Ti}(t) = \frac{k_{Xi}}{\delta} = X_i + X_i^* + C_{1i} + C_{2i} + C_{1_{i+1}}$ and $M_T(t) = \frac{k_M}{\delta} = M + \sum_{i=1}^N C_{2i}$, we reduce the system above to bring it to form (1) as seen in Table 4, with $c = \frac{C}{p_T}$. We make the following Assumptions for the system:

Assumption 10. All cycles have the same reaction constants, i.e., $\forall i \in [1, N]$, $k_{1i} = k_1, k_{2i} = k_2, a_{1i} = a_1, \beta_{1i} = a_2, d_{1i} = d_1, \beta_{2i} = d_2$. Then, $K_{m1i} = K_{m1}, K_{m2i} = K_{m2}$. Define $\lambda' = \frac{k_1 K_{m2}}{k_2 K_{m1}}$.

Assumption 11. $\forall t$ and $\forall i \in [1, N]$, $K_{m2} \gg X_i^*(t)$.

We now solve for $\underline{\Psi}$ by setting $(Br + f_1)_{3n\times 1} = 0$. Under Assumption 11, this is given by:

$$\begin{aligned}
&\underline{\Psi} \approx \left[\begin{array}{ccccc} ... & \frac{k_2}{k_1}\frac{M_T}{K_{m2}}\bar{X}_i^*, & \frac{M_T}{K_{m2}}\bar{X}_i^*, & \bar{X}_i^*, & ... \end{array}\right]^T_{3N\times 1}, \\
&\text{where } \bar{X}_i^* = \frac{\prod_{j=1}^i X_{Tj} Z}{b^i + (\sum_{j=1}^i (b^{i-j}\alpha_i(t)\prod_{k=1}^{j-1} X_{Tk}))Z} \text{ for } i \in [1, N-1], \\
&\text{and } \bar{X}_N^* = \frac{\prod_{j=1}^N X_{Tj} Z\left(1 - \frac{p_T}{X_{TN}} c(t)\right)}{b^N + (\sum_{j=1}^N (b^{N-j}\alpha_j(t)\prod_{k=1}^{j-1} X_{Tk}))Z} = \frac{\left(\frac{\prod_{j=1}^N X_{Tj}}{b^N}\right) Z\left(1 - \frac{p_T}{X_{TN}} c(t)\right)}{1 + (\sum_{j=1}^N (b^{-j}\alpha_j(t)\prod_{k=1}^{j-1} X_{Tk}))Z}.
\end{aligned} \tag{79}$$

Here, $\alpha_j(t) \leq \left(\frac{X_{T_{j+1}}}{K_{m1}} + (\frac{k_2}{k_1}+1)\frac{M_T}{K_{m2}} + 1\right)$ for $j \in [1, N-1]$, $\alpha_N(t) = \left((\frac{k_2}{k_1}+1)\frac{M_T}{K_{m2}} + 1\right)$ and $b = \frac{M_T}{\lambda'} = \frac{M_T k_2 K_{m1}}{k_1 K_{m2}}$.

Solving for ϕ by setting $s(\underline{X}, v) = 0$, we have:

$$\begin{aligned} & k_{\text{on}} X_N^*(1-c) = k_{\text{off}} c, \\ & \text{i.e., } X_N^* - X_N^* c = k_D c, \\ & \text{i.e., } \phi = c = \frac{X_N^*}{k_D + X_N^*}. \end{aligned} \tag{80}$$

We can use (79) and (80) to find $\underline{\Gamma}$ as defined in Remark 1, and find that this satisfies Assumption 7. Note that this $\underline{\Gamma}$ differs from $\underline{\Psi}$ only in the last 3 terms, involving $\bar{X}_N^*$. Functions $\underline{\Psi}$ and ϕ satisfy Assumptions 5 and 6. Further, from Table 4, we see that matrices T, Q, M and P satisfy Assumption 4, and functions f_0 and $\underline{r}$ satisfy Assumption 8. We further assume that Assumptions 3 and 9 are satisfied for this system. Thus, Theorems 1, 2 and 3 can be applied to this system.

Results: (i) Retroactivity to the input: Since $S_1 = 0$ from Table 4, under Claim 1, $h_2 = 0$. Further, $|R\underline{\Gamma}| \approx \frac{X_{T1}Z}{K_{m1}}\frac{b}{(b+a_1 Z)}$, and thus, to make h_1 small, we must have small $\frac{X_{T1}}{K_{m1}}$. For the final term, we see that $T^{-1}M = \begin{bmatrix} 1 & 0 & ... & 0 \end{bmatrix}$ and $T^{-1}MQ^{-1}P = 0$. Since $T^{-1}M$ only has an entry on the first term, and since $\frac{\partial \underline{\Gamma}}{\partial \overline{U}}$ and $\frac{\partial \underline{\Psi}}{\partial \overline{U}}$ differ only in the last 3 terms, we can compute the final term using (79). This gives the following expression:

$$\left| \left(T^{-1}M \frac{\partial \underline{\Gamma}(U)}{\partial U} + T^{-1}MQ^{-1}P \frac{\partial \phi}{\partial \underline{X}}\bigg|_{\underline{X}=\underline{\Gamma}(U)} \frac{\partial \underline{\Gamma}(U)}{\partial U} \right) \dot{U} \right| = \frac{X_{T1}}{K_{m1}} \frac{b^2}{(b+a_1 Z)^2} |\dot{Z}|.$$

Thus, for a small retroactivity to the input, $\frac{X_{T1}}{K_{m1}}$ must be small.

(ii) Retroactivity to the output: Since $S_1 = 0$, $h_2 = 0$. Further, $T^{-1}MQ^{-1}P = 0$, and thus $\bar{h}_3 = 0$. For $\bar{h}_1$ to be small, since $S_2 = 0$, we must have a small S_3. From Table 4, $S_3 = \frac{p_T}{X_{TN}}, \frac{\delta p_T}{a_2 M_T}$. Thus, if $X_{TN}, \frac{a_2 M_T}{\delta} \gg p_T$, $\bar{h}_1$ is small. Thus, for a small retroactivity to the output, X_{TN} and M_T must be large.

(iii) Input-output relationship: From (79), we see that

$$I\underline{\Gamma}_{is}(u) = I\underline{\Psi}(U_{is}, 0) \approx \frac{\left(\frac{\prod_{j=1}^N X_{Tj}}{b^N}\right)\frac{Z_{is}}{X_{T1}}}{1 + (\sum_{j=1}^N (b^{-j} a_j(t) \prod_{k=1}^{j-1} X_{Tk}))Z_{is}}. \tag{81}$$

Note that $b = \frac{M_T}{\lambda'}$ and $\prod_{j=1}^{i-1} X_{Tj}$ are constants, and the linear gain is $\frac{\lambda'^N \prod_{j=1}^{i-1} X_{Tj}}{M_T^N}$.

The upper bound for $a_i(t) = \left(\frac{\bar{X}_{i+1}(t)}{K_{m1}} + (\frac{k_2}{k_1}+1)\frac{M_T}{K_{m2}} + 1\right), i \in [1, N]$, is given by seeing that the maximum value for $\bar{X}_{i+1}$ is $X_{T_{i+1}}$. Let the maximum value of $Z(t)$ for which the input-output relationship is approximately linear be $Z_{\max}$. We then have:

$$(\sum_{i=1}^N (b^{-i} a_i \prod_{j=1}^{i-1} X_{Tj}))Z_{is} \leq \underbrace{\left(\sum_{i=1}^N (b^{-i} \left(\frac{X_{T_{i+1}}}{K_{m1}} + (\frac{k_2}{k_1}+1)\frac{M_T}{K_{m2}} + 1\right) \prod_{j=1}^{i-1} X_{Tj})\right)}_{\epsilon_3} Z_{\max},.$$

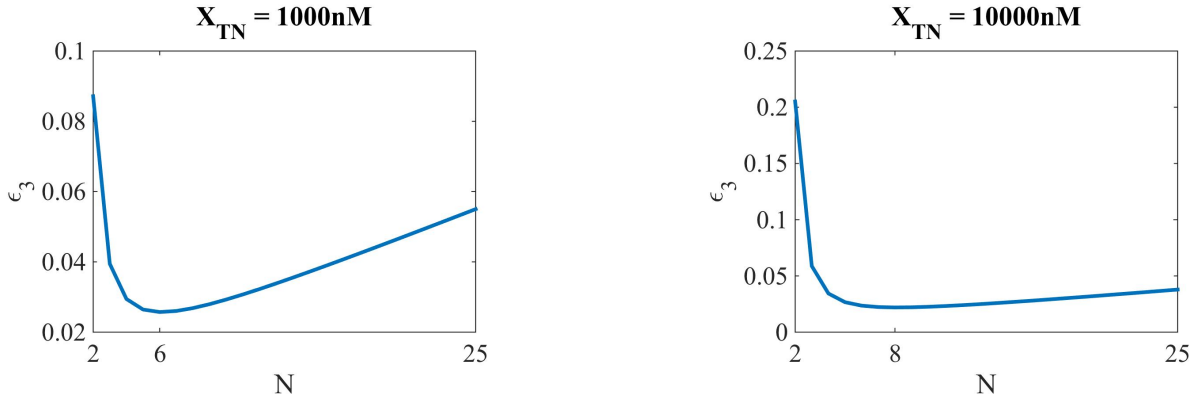
where $b = \frac{M_T}{\lambda'}$. Thus, for the input-output relationship to not saturate, $\epsilon_3 Z_{\max}$ must be small. To maximize $Z_{\max}$, the range in which the input-output relationship is linear, we must then minimize ϵ_3. We see that, to make ϵ_3 small, we must have a large b and small $X_{T_{i+1}}$. Since, to satisfy (ii), we saw before that X_{TN} must be large, we have $X_{T_{i+1}} \leq X_{TN}$. However, as seen from the expression of $I\underline{\Gamma}_{is}$, increasing b also decreases the input-output gain. For simplicity, the next arguments are made to achieve unit gain for the original input $Z_{is}(t)$ and output $X_{N,is}^*(t)$. For unit gain, $b^N = \prod_{j=1}^N X_{Tj}$. Since $X_{Tj} \leq X_{TN}, j \in [2, N]$, the maximum possible $b = \left(X_{T1} X_{TN}^{N-1}\right)^{\frac{1}{N}}$, which occurs when $X_{Tj} = X_{TN}, j \in [2, N]$. Thus, following this argument, for unit gain and maximum linear range of the input for any N, we have

$X_{Tj} = X_{TN}, j \in [2, N]$ and $b = \frac{M_T}{\lambda} = \left(X_{T1} X_{TN}^{N-1}\right)^{\frac{1}{N}}$. Substituting $M_T = \lambda X_{T1}^{\frac{1}{N}} X_{TN}^{\frac{N-1}{N}}$, and using the geometric series sum, we obtain the following expression for ϵ_3:

$$\epsilon_3 = \underbrace{\frac{1}{K_{m1}}\left(\frac{X_{TN}}{X_{T1}}\right)^{\frac{1}{N}} + \frac{1}{X_{T1}^{\frac{1}{N}} X_{TN}^{\frac{N-1}{N}}}}_{(1)} + (\frac{k_2}{k_1}+1)\frac{\lambda}{K_{m2}}$$
$$+ \underbrace{\left(\frac{X_{T1}}{X_{TN}K_{m1}} + (\frac{k_2}{k_1}+1)\frac{\lambda}{K_{m2}}\left(\frac{X_{T1}}{X_{TN}}\right)^{1+\frac{1}{N}} + \frac{X_{T1}}{X_{TN}^2}\right)}_{(2a)} \cdot \underbrace{\left(\frac{\frac{X_{TN}}{X_{T1}} - \left(\frac{X_{TN}}{X_{T1}}\right)^{\frac{2}{N}}}{\left(\frac{X_{TN}}{X_{T1}}\right)^{\frac{1}{N}} - 1}\right)}_{(2b)} \tag{82}$$
$$+ \underbrace{\frac{\lambda(\frac{k_2}{k_1}+1)}{K_{m2}}\left(\frac{X_{T1}}{X_{TN}}\right)^{\frac{1}{N}}}_{(2c)} + \frac{1}{X_{TN}}.$$

Starting from $N = 2$, we see that since $X_{T1} < X_{TN}$, term (1) decreases with N, terms (2a), (2b) and (2c) increase with N and as $N \to \infty$, $\epsilon_3 \to \infty$. The function ϵ_3 is continuous, and therefore, there exists an optimal number of cycles $\bar{N}$ for which the linear operating range of the input, $Z_{\max}$ is maximized.

The final condition that the cascade must satisfy to satisfy Def. 1 ϵ_3 to be small, so that $m = 1$ as defined in requirement (iii) of Def. 1. As discussed above, there is an optimal $\bar{N}$ at which ϵ_3 is minimized, all other parameters remaining the same. We see from Fig. 10, that with load, the number of cycles needed increase, since X_{TN} increases as load p_T is increased. Note that, it may not be necessary to have $\bar{N}$ cycles to achieve a desirable result, i.e., a sufficiently large operating range. However, it is possible that no N is capable of producing linearity for the desired operating range, since ϵ_3 is bounded below.



(a) **(b)**

Fig 10. Figures showing the variation of ϵ_3 with N, for different X_{TN}. Parameter values are: $K_{m1} = K_{m2} = 300nM$, $k_1 = k_2 = 600s^{-1}$, $\lambda = 1$, (a) $X_{TN} = 1000nM$, where resulting $\bar{N} = 6$ and (b) $X_{TN} = 10000nM$, where resulting $\bar{N} = 8$.

5.5.1 Simulation results for other cascades

Phosphotransfer + single cycle

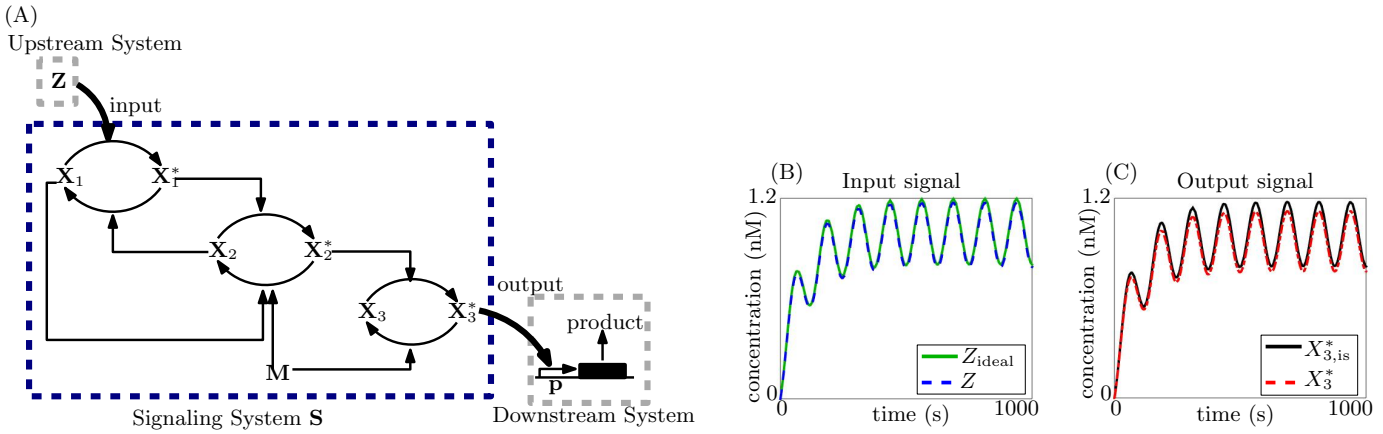


Fig 11. Tradeoff between small retroactivity to the input and attenuation of retroactivity to the output is overcome by a cascade of a phosphotransfer system with a single phosphorylation cycle. (A) Cascade of a phosphotransfer system that receives its input through a kinase Z phosphorylating the phosphate donor, and a phosphorylation cycle: Z phosphorylates X_1 to X_1^*, X_1^* transfers the phosphate group in a reversible reaction to X_2. X_2^* further acts as the kinase for X_3, phosphorylating it to X_3^*, which is the output, acting on sites p in the downstream system, which is depicted as a gene expression system here. Both X_2^* and X_3^* are dephosphorylated by phosphatase M. (B), (C) Simulation results for ODE model (83). Simulation parameters[1]:
$k(t) = 0.01(1 + sin(0.05t))nM.s^{-1}$, $\delta = 0.01s^{-1}$, $a_1 = a_2 = d_3 = a_4 = a_5 = a_6 = 18nM^{-1}s^{-1}$,
$d_1 = d_2 = a_3 = d_4 = d_5 = d_6 = 2400s^{-1}$, $k_1 = k_4 = k_5 = k_6 = 600s^{-1}$. (B) Effect of retroactivity to the input: for the ideal input Z_{ideal}, system is simulated with $X_{T1} = X_{T2} = X_{T3} = M_T = p_T = 0$; for actual input Z, system is simulated with $X_{T1} = 3nM$, $X_{T2} = 1200nM$, $X_{T3} = 1200nM$, $M_T = 3nM, p_T = 100nM$. (C) Effect of retroactivity to the output: for the isolated output $X^*_{3,\text{is}}$, system is simulated with $X_{T1} = 3nM$, $X_{T2} = 1200nM$, $X_{T3} = 1200nM$, $M_T = 3nM, p_T = 0$; for the actual output X_3^*, system is simulated with $X_{T1} = 3nM$, $X_{T2} = 1200nM$, $X_{T3} = 1200nM$, $M_T = 3nM, p_T = 100nM$.

Equations:

$$
\begin{aligned}
\dot{Z} &= k(t) - \delta Z - a_1 Z X_1 + (d_1 + k_1)C_1,\\
\dot{X}_1 &= k_{X_1} - \delta X_1 - a_1 Z X_1 + d_1 C_1 + a_3 C_2 - d_3 X_1 X_2^*,\\
\dot{C}_1 &= a_1 Z X_1 - (d_1 + k_1)C_1 - \delta C_1,\\
\dot{X}_1^* &= k_1 C_1 - a_2 X_1^* X_2 + d_2 C_2 - \delta X_1^*,\\
\dot{X}_2 &= k_{X_2} - \delta X_2 - a_2 X_1^* X_2 + d_2 C_2 + k_5 C_5,\\
\dot{C}_2 &= a_2 X_1^* X_2 + d_3 X_1 X_2^* - (d_2 + a_3)C_2 - \delta C_2,\\
\dot{X}_2^* &= a_3 C_2 - d_3 X_1 X_2^* - a_4 X_2^* X_3 + (d_4 + k_4)C_4 - a_5 X_2^* M + d_5 C_5 - \delta X_2^*,\\
\dot{X}_3 &= k_{X_3} - \delta X_3 - a_4 X_2^* X_3 + d_4 C_4 + k_6 C_6,\\
\dot{C}_4 &= a_4 X_2^* X_3 - (d_4 + k_4)C_4 - \delta C_4,\\
\dot{X}_3^* &= k_4 C_4 - a_6 X_3^* M + d_6 C_6 - \delta X_3^* - k_{\text{on}} X_3^* p + k_{\text{off}} C,\\
\dot{M} &= k_M - \delta M - a_5 X_2^* M + (d_5 + k_5)C_5 - a_6 X_3^* M + (d_6 + k_6)C_6,\\
\dot{C}_5 &= a_5 X_2^* M - (d_5 + k_5)C_5 - \delta C_5,\\
\dot{C}_6 &= a_6 X_3^* M - (d_6 + k_6)C_6 - \delta C_6,\\
\dot{C} &= k_{\text{on}} X_3^* p - k_{\text{off}} C - \delta C.
\end{aligned}
\tag{83}
$$

Single + Double cycle

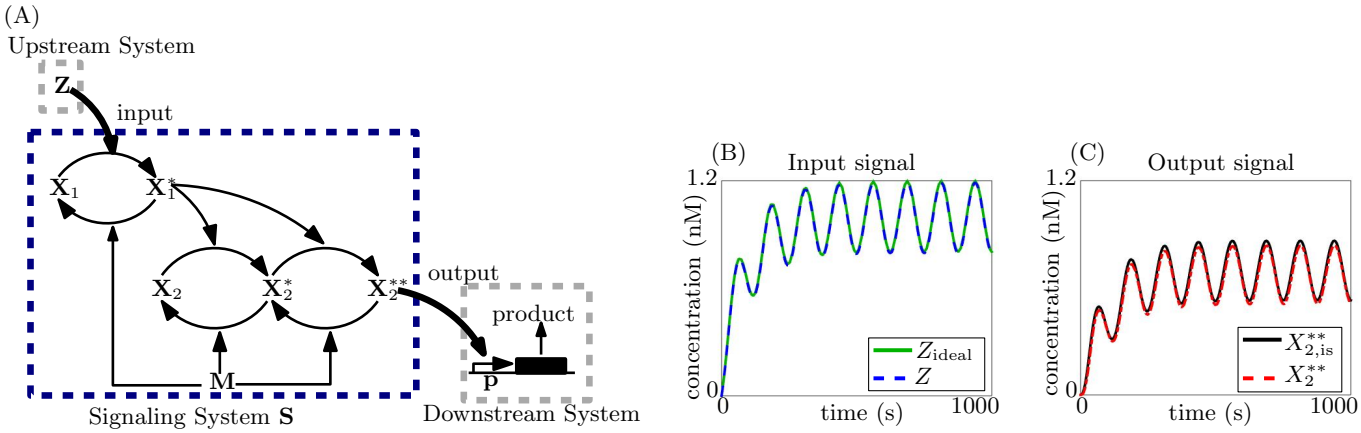


Fig 12. Tradeoff between small retroactivity to the input and attenuation of retroactivity to the output is overcome by a cascade of a single phosphorylation cycle and a double phosphorylation cycle. (A) Cascade of a a single phosphorylation and a double phosphorylation cycle with input kinase Z: Z phosphorylates X_1 to X_1^*, X_1^* further acts as the kinase for X_2, phosphorylating it to X_2^* and X_2^{**}, which is the output, acting on sites p in the downstream system, which is depicted as a gene expression system here. All phosphorylated proteins X_1^*, X_2^* and X_2^{**} are dephosphorylated by phosphatase M. (B), (C) Simulation results for ODE model (84). Simulation parameters[1]: $k(t) = 0.01(1 + sin(0.05t))nM.s^{-1}$, $\delta = 0.01s^{-1}$, $a_1 = a_2 = a_3 = a_4 = a_5 = a_6 = 18nM^{-1}s^{-1}$, $d_1 = d_2 = d_3 = d_4 = d_5 = d_6 = 2400s^{-1}$, $k_1 = k_2 = k_3 = k_4 = k_5 = k_6 = 600s^{-1}$. (B) Effect of retroactivity to the input: for the ideal input Z_{ideal}, system is simulated with $X_{T1} = X_{T2} = X_{T3} = M_T = p_T = 0$; for actual input Z, system is simulated with $X_{T1} = 3nM$, $X_{T2} = 1200nM$, $M_T = 9nM, p_T = 100nM$. (C) Effect of retroactivity to the output: for the isolated output $X^*_{2,\text{is}}$, system is simulated with $X_{T1} = 3nM$, $X_{T2} = 1200nM$, $M_T = 9nM, p_T = 0$; for the actual output X_2^*, system is simulated with $X_{T1} = 3nM$, $X_{T2} = 1200nM$, $M_T = 9nM, p_T = 100nM$.

Equations:

$$
\begin{aligned}
\dot{Z} &= k(t) - \delta Z - a_1 Z X_1 + (d_1 + k_1)C_1,\\
\dot{X}_1 &= k_{X_1} - \delta X_1 - a_1 Z X_1 + d_1 C_1 + k_2 C_2,\\
\dot{C}_1 &= a_1 Z X_1 - (d_1 + k_1)C_1 - \delta C_1,\\
\dot{X}_1^* &= k_1 C_1 - a_2 X_1^* M + d_2 C_2 - a_3 X_1^* X_2 + (d_3 + k_3)C_3 - a_4 X_1^* X_2^* + (d_4 + k_4)C_4 - \delta X_1^*,\\
\dot{M} &= k_M - \delta M - a_2 X_1^* M + (d_2 + k_2)C_2 - a_5 X_2^* M + (d_5 + k_5)C_5\\
&\quad - a_6 X_2^{**} M + (d_6 + k_6)C_6,\\
\dot{C}_2 &= a_2 X_1^* M - (d_2 + k_2)C_2 - \delta C_2,\\
\dot{X}_2 &= k_{X_2} - \delta X_2 - a_3 X_1^* X_2 + d_3 C_3 + k_5 C_5,\\
\dot{C}_3 &= a_3 X_1^* X_2 - (d_3 + k_3)C_3 - \delta C_3,\\
\dot{X}_2^* &= k_3 C_3 - a_4 X_1^* X_2^* + d_4 C_4 - a_5 X_2^* M + d_5 C_5 + k_6 C_6 - \delta X_2^*,\\
\dot{C}_4 &= a_4 X_1^* X_2^* - (d_4 + k_4)C_4 - \delta C_4,\\
\dot{X}_2^{**} &= k_4 C_4 - a_6 X_2^{**} M + d_6 C_6 - k_{\text{on}} X_2^{**} p + k_{\text{off}} C - \delta X_2^{**},\\
\dot{C}_5 &= a_5 X_2^* M - (d_5 + k_5)C_5 - \delta C_5,\\
\dot{C}_6 &= a_6 X_2^{**} M - (d_6 + k_6)C_6 - \delta C_6,\\
\dot{C} &= k_{\text{on}} X_2^{**} p - k_{\text{off}} C - \delta C.
\end{aligned}
\tag{84}
$$

5.6 Phosphotransfer with autophosphorylation

The reactions for this system are then:

$$X_1 \underset{k(t)}{\overset{\delta}{\rightleftharpoons}} \phi, \qquad X_2 \underset{k_{X_2}}{\overset{\delta}{\rightleftharpoons}} \phi, \tag{85}$$

$$M \underset{k_M}{\overset{\delta}{\rightleftharpoons}} \phi, \qquad X_1^*, C_1, X_2^*, C_3, C \xrightarrow{\delta} \phi, \tag{86}$$

$$X_1 \xrightarrow{\pi_1} X_1^*, \qquad X_1^* + X_2 \underset{d_1}{\overset{a_1}{\rightleftharpoons}} C_1 \underset{a_2}{\overset{d_2}{\rightleftharpoons}} X_1 + X_2^*, \tag{87}$$

$$X_2^* + M \underset{d_3}{\overset{a_3}{\rightleftharpoons}} C_3 \xrightarrow{k_3} X_2 + M, \qquad X_2^* + p \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} C. \tag{88}$$

The ODEs based on the reaction rate equations are:

$$\begin{aligned}
\dot{X}_1 &= k(t) - \delta X_1 - \pi_1 X_1 + d_2 C_1 - a_2 X_2^* X_1, & X_1(0) &= 0,\\
\dot{X}_1^* &= \pi_1 X_1 - a_1 X_1^* X_2 + d_1 C_1 - \delta X_1^*, & X_1^*(0) &= 0,\\
\dot{C}_1 &= -\delta C_1 + a_1 X_1^* X_2 - (d_1 + d_2) C_1 + a_2 X_2^* X_1, & C_1(0) &= 0,\\
\dot{X}_2 &= k_{X_2} - \delta X_2 - a_1 X_1^* X_2 + d_1 C_1 + k_3 C_3, & X_2(0) &= \frac{k_{X_2}}{\delta},\\
\dot{X}_2^* &= -\delta X_2^* + d_2 C_1 - a_2 X_2^* X_1 - a_3 X_2^* M + d_3 C_3 - k_{\text{on}} X_2^* (p_T - C) + k_{\text{off}} C, & X_2^*(0) &= 0,\\
\dot{C}_3 &= -\delta C_3 + a_3 X_2^* M - (d_3 + k_3) C_3, & C_3(0) &= 0,\\
\dot{M} &= k_M - \delta M - a_3 X_2^* M + (d_3 + k_3) C_3, & M(0) &= \frac{k_M}{\delta},\\
\dot{C} &= k_{\text{on}} X_2^* (p_T - C) - k_{\text{off}} C - \delta C, & C(0) &= 0.
\end{aligned} \tag{89}$$

For system (89), define $X_{T2} = X_2 + X_2^* + C_1 + C_3 + C$, then $\dot{X}_{T2} = k_{X_2} - \delta X_{T2}, X_{T2} = \frac{k_{X_2}}{\delta}$. Thus, $X_{T2}(t) = \frac{k_{X_2}}{\delta}$ is a constant. Similarly, defining $M_T = M + C_3$ gives a constant $M_T(t) = \frac{k_M}{\delta}$. Thus, the variables $X_2 = X_{T2} - X_2^* - C_1 - C_3 - C$ and $M = M_T - C_3$ can be eliminated from the system. Further, we define $c = \frac{C}{p_T}$. This system is then:

$$\begin{aligned}
\dot{X}_1 &= k(t) - \delta X_1 - \pi_1 X_1 + d_2 C_1 - a_2 X_2^* X_1, & X_1(0) &= 0,\\
\dot{X}_1^* &= \pi_1 X_1 - a_1 X_1^* (X_{T2} - X_2^* - C_1 - C_3 - p_T c) + d_1 C_1 - \delta X_1^*, & X_1^*(0) &= 0,\\
\dot{C}_1 &= -\delta C_1 + a_1 X_1^* (X_{T2} - X_2^* - C_1 - C_3 - p_T c) - (d_1 + d_2) C_1 + a_2 X_2^* X_1, & C_1(0) &= 0,\\
\dot{X}_2^* &= -\delta X_2^* + d_2 C_1 - a_2 X_2^* X_1 - a_3 X_2^* (M_T - C_3) + d_3 C_3 - k_{\text{on}} X_2^* p_T (1 - c) + k_{\text{off}} C, & X_2^*(0) &= 0,\\
\dot{C}_3 &= -\delta C_3 + a_3 X_2^* (M_T - C_3) - (d_3 + k_3) C_3, & C_3(0) &= 0,\\
\dot{c} &= k_{\text{on}} X_2^* (1 - c) - k_{\text{off}} c - \delta c, & c(0) &= 0.
\end{aligned} \tag{90}$$

Based on eqns. (90), we bring the system to form (1) as shown in Table 5. We now solve for the functions $\underline{\Psi}$ and ϕ as defined by Assumptions 5 and 6.

Solving for $\underline{X} = \underline{\Psi}$ by setting $(Br + f_1)_4 = 0$, we have:

$$\begin{aligned}
&(Br + f_1)_1 + (Br + f_1)_2 + (Br + f_1)_3 + (Br + f_1)_4 = 0 \implies\\
&\pi_1 X_1 - k_3 C_3 \approx 0, \text{ i.e., } C_3 \approx \frac{\pi_1}{k_3} X_1.
\end{aligned}$$

$$\begin{aligned}
&(Br + f_1)_4 = 0 \implies a_3 X_2^* (M_T - C_3) \approx (d_3 + k_3) C_3.\\
&\text{If } K_{m3} \gg X_2^*,\ X_2^* \approx \frac{\pi_1 K_{m3}}{k_3 M_T} X_1 = K X_1, \text{ where } K = \frac{\pi_1 K_{m3}}{k_3 M_T}.
\end{aligned}$$

U	X_1	v	c
$\underline{X}$	$\left[\begin{array}{cccc} X_1^* & C_1 & X_2^* & C_3 \end{array}\right]_{4\times 1}^T$	Y, I	X_2^*, $\left[\begin{array}{cccc} 0 & 0 & 1 & 0 \end{array}\right]_{1\times 4}$
G_1	$\max\left\{\frac{a_1 X_{T2}}{\delta}, \frac{d_1}{\delta}, \frac{d_2}{\delta}, \frac{a_2 X_{T1}}{\delta}, \frac{a_3 M_T}{\delta}, \frac{d_3}{\delta}, \frac{k_3}{\delta}\right\}$	G_2	$\max\left\{\frac{k_{\text{on}} p_T}{\delta}, \frac{k_{\text{off}}}{\delta}\right\}$
$f_0(U, R\underline{X}, S_1 v, t)$	$k(t) - \delta X_1 - \delta C_1 - \delta X_1^*$	$s(\underline{X}, v)$	$\frac{1}{G_2}\left(k_{\text{on}} X_2^*(1-c) - k_{\text{off}} c - \delta c\right)$
$\underline{r}(U, \underline{X}, S_2 v)$	$\frac{1}{G_1}\left[\begin{array}{c} -\pi_1 X_1 + \delta X_1^*, \\ d_2 C_1 - a_2 X_2^* X_1 + \delta C_1 \end{array}\right]_{2\times 1}$		
$f_1(U, \underline{X}, S_3 v)$	$\frac{1}{G_1}\left[\begin{array}{c} -a_1 X_{T2} X_1^*(1 - \frac{C_1}{X_{T2}} - \frac{X_2^*}{X_{T2}} - \frac{C_3}{X_{T2}} - \frac{p_T}{X_{T2}} c) + d_1 C_1, \\ a_1 X_{T2} X_1^*(1 - \frac{C_1}{X_{T2}} - \frac{X_2^*}{X_{T2}} - \frac{C_3}{X_{T2}} - \frac{p_T}{X_{T2}} c) - d_1 C_1, \\ -\delta X_2^* + d_2 C_1 - a_2 X_2^* X_1 + a_3 X_2^* C_3 + d_3 C_3 - a_3 M_T (X_2^* + \frac{p_T \delta}{a_3 M_T} c), \\ -\delta C_3 + a_3 X_2^*(M_T - C_3) - (d_3 + k_3) C_3 \end{array}\right]_{4\times 1}$		
A	$\left[\begin{array}{cc} 1 & 1 \end{array}\right]_{1\times 2}$	D	1
B	$\left[\begin{array}{cc} -1 & 0 \\ 0 & -1 \\ 0 & 0 \\ 0 & 0 \end{array}\right]_{4\times 2}$	C	$\left[\begin{array}{c} 0 \\ 0 \\ -p_T \\ 0 \end{array}\right]_{4\times 1}$
R	$\left[\begin{array}{cccc} 1 & 1 & 0 & 0 \end{array}\right]_{1\times 4}$	S_1	0
S_2	0	S_3	$\frac{p_T}{X_{T2}}, \frac{p_T \delta}{a_3 M_T}$
T	$\mathbb{I}_{2\times 2}$	M	$\left[\begin{array}{cccc} 1 & 1 & 0 & 0 \end{array}\right]_{1\times 4}$
Q	$\mathbb{I}_{4\times 4}$	P	$\left[\begin{array}{cccc} 0 & 0 & p_T & 0 \end{array}\right]_{4\times 1}^T$

Table 5. System variables, functions and matrices for a phosphotransfer system with autophosphorylation brought to form (1).

$$(Br+f_1)_1+(Br+f_1)_2=0 \implies \pi_1 X_1 - d_2 C_1 + a_2 X_2^* X_1 \approx 0,$$
$$\text{i.e., } C_1 \approx \frac{a_2 K}{d_2} X_1^2 + \frac{\pi_1}{d_2} X_1.$$

$$(Br+f_1)_2=0 \implies$$
$$-C_1 + a_1 X_1^* X_{T2}\left(1 - \frac{C_1}{X_{T2}} - \frac{X_2^*}{X_{T2}} - \frac{C_3}{X_{T2}} - \frac{p_T}{X_{T2}}c\right) - (d_1+d_2)C_1 + a_2 X_2^* X_1 = 0.$$
$$\text{If } (d_1+d_2) \gg a_1 X_1^*,\ X_1^* \approx \frac{(d_1+d_2)C_1 - a_2 K X_1^2}{a_1 X_{T2}} \approx \frac{d_1 a_2 K}{a_1 d_2 X_{T2}} X_1^2 + \frac{\pi_1 (d_1+d_2)}{a_1 d_2 X_{T2}} X_1.$$

Thus, we have the function $\underline{\Psi}(U, v)$:

$$\underline{\Psi} \approx \begin{bmatrix} \frac{d_1 a_2 K}{a_1 d_2 X_{T2}} X_1^2 + \frac{\pi_1 (d_1+d_2)}{a_1 d_2 X_{T2}} X_1, \\ \frac{a_2 K}{d_2} X_1^2 + \frac{\pi_1}{d_2} X_1, \\ K x_1, \\ \frac{\pi_1}{k_3} X_1 \end{bmatrix}_{4\times 1}, \text{ where } K = \frac{\pi_1 K_{m3}}{k_3 M_T}. \tag{91}$$

Solving for ϕ by setting $s(\underline{X}, v) = 0$, we have:

$$\begin{aligned} &k_{\text{on}} X_2^* (1-c) - k_{\text{off}} c - c = 0. \\ &\text{Under Assumption 1, } X_2^* - X_2^* c \approx k_D c, \\ &\text{i.e., } \phi = c \approx \frac{X_2^*}{X_2^* + k_D}. \end{aligned} \tag{92}$$

Again, we find $\underline{\Gamma}$ from (91) and (92) under Remark 1. This system satisfies Assumptions 3-9. Theorems 1-3 can then be applied.

Results: (i) Retroactivity to input: Under Theorem 1, we see that since $S_1 = 0$ from Table 5, $h_2 = 0$. Further, $|R\underline{\Gamma}(U)| \approx \frac{d_1 a_2 K}{a_1 d_2 X_{T2}} X_1^2 + \frac{\pi_1 (d_1+d_2)}{a_1 d_2 X_{T2}} X_1 + \frac{a_2 K}{d_2} X_1^2 + \frac{\pi_1}{d_2} X_1$. To compute the final term h_3, we see that:

$$\left| \left(T^{-1} M \frac{\partial \underline{\Gamma}(U)}{\partial U} + T^{-1} M Q^{-1} P \frac{\partial \phi}{\partial \underline{X}}\bigg|_{\underline{X}=\underline{\Gamma}(U)} \frac{\partial \underline{\Gamma}(U)}{\partial U} \right) \right| \approx$$
$$\frac{2 d_1 a_2 K}{a_1 d_2 X_{T2}} X_1 + \frac{\pi_1 (d_1+d_2)}{a_1 d_2 X_{T2}} + \frac{2 a_2 K}{d_2} X_1 + \frac{\pi_1}{a_2}.$$

Thus, for a small retroactivity to the input, terms $\frac{2 d_1 a_2 K}{a_1 d_2 X_{T2}}$, $\frac{\pi_1 (d_1+d_2)}{a_1 d_2 X_{T2}}$, $\frac{2 a_2 K}{d_2}$ and $\frac{\pi_1}{d_2}$ must be small. However, these terms cannot be made smaller by varying concentrations alone. Thus the retroactivity to the input depends on the reaction rate parameters of the system, and is harder to tune.

(ii) Retroactivity to output: Using Claim 2, we see from Table 5 that $S_1 = 0$, thus $h_2 = 0$. Further, $T^{-1} M Q^{-1} P = 0$, thus $\bar{h}_3 = 0$. For the last term, $\bar{h}_1$, we see that $S_2 = 0$ and thus, for small $\bar{h}_1$ implying small retroactivity to the output, we must have a small $S_3 = \frac{p_T}{X_{T2}}, \frac{p_T \delta}{a_3 M_T}$.

(iii) Input-output relationship: From (91), we see that

$$Y_{is} = I\underline{X}_{is} \approx I\underline{\Gamma}_{is} = I\underline{\Psi}(U_{is}, 0) \approx \frac{\pi_1 K_{m3}}{k_3 M_T} X_{1,is}. \tag{93}$$

Thus, the dimensionless output X_2^* varies linearly with the dimensionless input X_1, i.e., $m = 1$ and $K = \frac{\pi_1 K_{m3}}{k_3 M_T}$.

5.7 Single cycle with substrate input

The reactions for this system are:

$$X \underset{k(t)}{\overset{\delta}{\rightleftharpoons}} \phi, \qquad Z \underset{k_Z}{\overset{\delta}{\rightleftharpoons}} \phi, \tag{94}$$

$$M \underset{k_M}{\overset{\delta}{\rightleftharpoons}} \phi, \qquad C_1, C_2, X^*, C \xrightarrow{\delta} \phi, \tag{95}$$

$$X + Z \underset{d_1}{\overset{a_1}{\rightleftharpoons}} C_1 \xrightarrow{k_1} X^* + Z, \qquad X^* + M \underset{d_2}{\overset{a_2}{\rightleftharpoons}} C_2 \xrightarrow{k_2} X + M, \tag{96}$$

$$X^* + p \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} C. \tag{97}$$

The corresponding ODEs based on the reaction rate equations are then:

$$\begin{aligned}
\dot{X} &= k(t) - \delta X - a_1 X Z + d_1 C_1 + k_2 C_2, && X(0) = 0,\\
\dot{X}^* &= -\delta X^* + k_1 C_1 - a_2 X^* M + d_2 C_2 - k_{\text{on}} X^* (p_T - C) + k_{\text{off}} C, && X^*(0) = 0,\\
\dot{C}_1 &= a_1 X Z - (d_1 + k_1) C_1 - \delta C_1, && C_1(0) = 0,\\
\dot{C}_2 &= a_2 X^* M - (d_2 + k_2) C_2 - \delta C_2, && C_2(0) = 0,\\
\dot{Z} &= k_Z - \delta Z - a_1 X Z + (k_1 + d_1) C_1, && Z(0) = \frac{k_Z}{\delta},\\
\dot{M} &= k_M - \delta M - a_2 X^* M + (d_2 + k_2) C_2, && M(0) = \frac{k_M}{\delta},\\
\dot{C} &= k_{\text{on}} X^* (p_T - C) - k_{\text{off}} C - \delta C, && C(0) = 0.
\end{aligned} \tag{98}$$

Let $Z_T = Z + C_1$. Then, from the ODEs (98) and the initial conditions, we see that $\dot{Z}_T = k_Z - \delta Z_T$, $Z_T(0) = \frac{k_Z}{\delta}$. Thus, $Z_T(t) = \frac{k_Z}{\delta}$ is a constant. Similarly, defining $M_T = M + C_2$ gives a constant $M_T(t) = \frac{k_M}{\delta}$. The variables $Z = Z_T - C_1$ and $M = M_T - C_2$ can then be eliminated from the system. Further, we define $c = \frac{C}{p_T}$. The reduced system is then:

$$\begin{aligned}
\dot{X} &= k(t) - \delta X - a_1 X (Z_T - C_1) + d_1 C_1 + k_2 C_2, && X(0) = 0,\\
\dot{X}^* &= -\delta X^* + k_1 C_1 - a_2 X^* (M_T - C_2) + d_2 C_2 - k_{\text{on}} X^* p_T (1 - c) + k_{\text{off}} p_T c, && X^*(0) = 0,\\
\dot{C}_1 &= a_1 X (Z_T - C_1) - (d_1 + k_1) C_1 - \delta C_1, && C_1(0) = 0,\\
\dot{C}_2 &= a_2 X^* (M_T - C_2) - (d_2 + k_2) C_2 - \delta C_2, && C_2(0) = 0,\\
\dot{c} &= k_{\text{on}} X^* (1 - c) - k_{\text{off}} c - \delta c, && c(0) = 0.
\end{aligned} \tag{99}$$

Based on the system of ODEs (99), we bring this system to form (1) as shown in Table 6. We now solve for the functions $\underline{\Psi}$ and ϕ as defined by Assumptions 5 and 6.

Solving for $\underline{X} = \underline{\Psi}$ by setting $(Br + f_1)_{3\times 1} = 0$, we have:

$$\begin{aligned}
&(Br + f_1)_2 = 0 \implies a_1 X (Z_T - C_1) = (d_1 + k_1 + \delta)\, C_1,\\
&\text{since } (d_1 + k_1) \gg \delta \text{ under Assumption 1,}\\
&X Z_T - X C_1 \approx K_{m1} C_1,\\
&\text{i.e., } C_1 \approx \frac{X}{X + K_{m1}}.\\
&\text{For } K_{m1} \gg X,\ C_1 \approx \frac{X}{K_{m1}}.
\end{aligned} \tag{100}$$

U	X	v	c
$\underline{X}$	$\begin{bmatrix} X^* & C_1 & C_2 \end{bmatrix}^T_{3\times1}$	$Y,\ I$	$X^*, \begin{bmatrix} 1 & 0 & 0 \end{bmatrix}_{1\times3}$
G_1	$\max\left\{\frac{a_1Z_T}{\delta}, \frac{d_1}{\delta}, \frac{k_1}{\delta}, \frac{a_2M_T}{\delta}, \frac{d_2}{\delta}, \frac{k_2}{\delta}\right\}$	G_2	$\max\left\{\frac{k_{\text{on}}p_T}{\delta}, \frac{k_{\text{off}}}{\delta}\right\}$
$f_0(U, R\underline{X}, S_1v, t)$	$k(t) - \delta X - \delta X^* - \delta C_1 - \delta C_2 - \delta p_T c$	$s(\underline{X}, v)$	$\frac{1}{G_2}\left(k_{\text{on}}X^*(1-c) - k_{\text{off}}c - \delta c\right)$
$\underline{r}(U, \underline{X}, S_2v)$	$\frac{1}{G_1}\begin{bmatrix} \delta(X^* + p_Tc), & -a_1X(Z_T - C_1) + d_1C_1 + \delta C_1, & k_2C_2 + \delta C_2 \end{bmatrix}^T_{3\times1}$		
$f_1(U, \underline{X}, S_3v)$	$\frac{1}{G_1}\begin{bmatrix} k_1C_1 - a_2X(M_T - C_2) + d_2C_2, & -k_1C_1, & a_2X^*(M_T - C_2) - d_2C_2 \end{bmatrix}^T_{3\times1}$		
A	$\begin{bmatrix} 1 & 1 & 1 \end{bmatrix}_{1\times3}$	D	1
B	$\begin{bmatrix} -1 & 0 & 0 \\ 0 & -1 & 0 \\ 0 & 0 & -1 \end{bmatrix}_{3\times3}$	C	$\begin{bmatrix} -p_T \\ 0 \\ 0 \end{bmatrix}_{3\times1}$
R	$\begin{bmatrix} 1 & 1 & 1 \end{bmatrix}_{1\times3}$	S_1	p_T
S_2	p_T	S_3	0
T	1	M	$\begin{bmatrix} 1 & 1 & 1 \end{bmatrix}_{1\times3}$
Q	$\mathbb{I}_{3\times3}$	P	$\begin{bmatrix} p_T & 0 & 0 \end{bmatrix}^T_{3\times1}$

Table 6. System variables, functions and matrices for a single phosphorylation cycle with substrate as input brought to form (1).

$$
\begin{aligned}
&(Br + f_1)_3 = 0 \implies a_2X^*(M_T - C_2) = (d_2 + k_2 + \delta)\, C_2,\\
&\text{since } (d_2 + k_2) \gg \delta \text{ under Assumption 1,}\\
&X^*M_T - X^*C_2 = K_{m2}C_2,\\
&\text{i.e., } C_2 = \frac{X^*}{X^* + K_{m2}}.\\
&\text{If } K_{m2} \gg X^*,\ C_2 \approx \frac{X^*}{K_{m2}}.
\end{aligned}
\tag{101}
$$

$$
\begin{aligned}
&(Br + f_1)_1 = 0 \implies -\delta X^* - \delta p_T c + k_1C_1 - k_2C_2 = 0.\\
&\text{Using (100) and (101), we have: } \frac{k_1X}{K_{m1}} - \frac{k_2X^*}{K_{m2}} - \delta X^* - \delta p_T c \approx 0,\\
&\text{i.e., } X^* \approx \frac{\left(\frac{k_1Z_T}{K_{m1}}\right)}{\frac{k_2M_T}{K_{m2}} + \delta}X - \frac{\delta p_T}{\frac{k_2M_T}{K_{m2}} + \delta}c.
\end{aligned}
\tag{102}
$$

Thus, from equations (100)-(102), we have the function $\underline{\Psi}(U, v)$:

$$
\underline{\Psi} \approx \begin{bmatrix} \frac{\left(\frac{k_1Z_T}{K_{m1}}\right)}{\frac{k_2M_T}{K_{m2}} + \delta}X - \frac{\delta p_T}{\frac{k_2M_T}{K_{m2}} + \delta}c, & \frac{X}{K_{m1}}, & \frac{X}{K_{m2}}\left(\frac{\left(\frac{k_1Z_T}{K_{m1}}\right)}{\frac{k_2M_T}{K_{m2}} + \delta} - \frac{\delta p_T}{\frac{k_2M_T}{K_{m2}} + \delta}c\right) \end{bmatrix}^T. \tag{103}
$$

Solving for $v = \phi(\underline{X})$ by setting $s(\underline{X}, v) = 0$, we have:

$$\begin{aligned} &k_{\text{on}} X^*(1-c) = k_{\text{off}} c, \\ &\text{i.e., } X^* - X^* c = k_D c, \\ &\text{i.e., } \phi(\underline{X}) = c = \frac{X^*}{k_D + X^*}. \end{aligned} \tag{104}$$

Using (103) and (104), $\underline{\Gamma}$ can be found as described in Remark 1. We find that this satisfies Assumption 7. We then state the following claims without proof for this system:

Claim 5. *For the matrix B and functions r, f_1 and s defined in Table 6, Assumption 3 is satisfied for this system.*

Claim 6. *For the functions f_0 and $\underline{r}$ and matrices R, S_1 and A defined in Table 6, and the functions $\underline{\Gamma}$ and ϕ as found above, Assumption 9 is satisfied for this system.*

For matrices T, Q, M and P as seen in Table 6, we see that Assumption 4 is satisfied. For functions f_0 and $\underline{r}$ defined in Table 6, Assumption 8 is satisfied. Further, for $\underline{\Psi}$ and ϕ defined by (103) and (104), Assumptions 5, 6 and 7 are satisfied. Thus, Theorems 1, 2 and 3 can be applied to this system.

Results: (i) Retroactivity to the input: From Table 6, we see that R and S_1 cannot be made small by changing system variables. Under Claim 1, therefore, retroactivity to the input cannot be made small.

(ii) Retroactivity to the output: From Table 6, we see that S_1 and S_2 cannot be made small. Under Claim 2, therefore, retroactivity to the output cannot be made small.

(iii) Input-output relationship: Using Theorem 3, we see that

$$Y_{is}(t) = I\underline{X}_{is} \approx I\underline{\Gamma}_{is} = I\underline{\Psi}(U_{is}, 0) \approx KX_{is}(t), \tag{105}$$

for $t \in [t_b, t_f]$ from (103), where $K = \begin{pmatrix} \frac{k_1 Z_T}{K_{m1}} \\ \frac{k_2 M_T}{K_{m2}} + \delta \end{pmatrix}$.

5.8 Double cycle with substrate input

The reactions for this system are:

$$X \underset{k(t)}{\overset{\delta}{\rightleftharpoons}} \phi, \qquad Z \underset{k_Z}{\overset{\delta}{\rightleftharpoons}} \phi, \tag{106}$$

$$M \underset{k_M}{\overset{\delta}{\rightleftharpoons}} \phi, \qquad C_1, C_2, C_3, C_4, X^*, X^{**}, C \xrightarrow{\delta} \phi, \tag{107}$$

$$X + Z \underset{d_1}{\overset{a_1}{\rightleftharpoons}} C_1 \xrightarrow{k_1} X^* + Z, \qquad X^* + M \underset{d_2}{\overset{a_2}{\rightleftharpoons}} C_2 \xrightarrow{k_2} X + M, \tag{108}$$

$$X^* + Z \underset{d_3}{\overset{a_3}{\rightleftharpoons}} C_3 \xrightarrow{k_3} X^{**} + Z, \qquad X^{**} + M \underset{d_4}{\overset{a_4}{\rightleftharpoons}} C_4 \xrightarrow{k_4} X^* + M, \tag{109}$$

$$X^{**} + p \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} C. \tag{110}$$

The ODEs based on the reaction rate equations are:

$$
\begin{aligned}
\dot{X} &= k(t) - \delta X - a_1 X Z + d_1 C_1 + k_2 C_2, && X(0) = 0,\\
\dot{X}^* &= -\delta X^* + k_1 C_1 - a_2 X^* M + d_2 C_2 - a_3 X^* Z + d_3 C_3 + k_4 C_4, && X^*(0) = 0,\\
\dot{X}^{**} &= -\delta X^{**} + k_3 C_3 - a_4 X^{**} M + d_4 C_4 - k_{\text{on}} X^{**}(p_T - C) + k_{\text{off}} C, && X^{**}(0) = 0,\\
\dot{Z} &= k_Z - \delta Z - a_1 X Z + (d_1 + k_1) C_1 - a_3 X^* Z + (d_3 + k_3) C_3, && Z(0) = \frac{k_Z}{\delta},\\
\dot{M} &= k_M - \delta M - a_2 X^* M + (d_2 + k_2) C_2 - a_4 X^{**} M + (d_4 + k_4) C_4, && M(0) = \frac{k_M}{\delta},\\
\dot{C}_1 &= a_1 X Z - (d_1 + k_1) C_1 - \delta C_1, && C_1(0) = 0,\\
\dot{C}_2 &= a_2 X^* M - (d_2 + k_2) C_2 - \delta C_2, && C_2(0) = 0,\\
\dot{C}_3 &= a_3 X^* Z - (d_3 + k_3) C_3 - \delta C_3, && C_3(0) = 0,\\
\dot{C}_4 &= a_4 X^{**} M - (d_4 + k_4) C_4 - \delta C_4, && C_4(0) = 0,\\
\dot{C} &= k_{\text{on}} Z^{**}(p_T - C) - k_{\text{off}} C - \delta C, && C(0) = 0.
\end{aligned}
\tag{111}
$$

Define $Z_T = Z + C_1 + C_3$. Then, the dynamics of Z_T, seen from (111), are: $\dot{Z}_T = k_Z - \delta Z_T$, $Z_T(0) = \frac{k_Z}{\delta}$. Thus, $Z_T(t) = \frac{k_Z}{\delta}$ is a constant at all time t. Similarly, for $M_T = M + C_2 + C_4$, $M_T(t) = \frac{k_M}{\delta}$ is a constant for all t. Thus, the variables $Z = Z_T - C_1 - C_2$ and $M = M_T - C_2 - C_4$ can be eliminated from the system. Further, we define $c = \frac{C}{p_T}$. The reduced system is then:

$$
\begin{aligned}
\dot{X} &= k(t) - \delta X - a_1 X(Z_T - C_1 - C_2) + d_1 C_1 + k_2 C_2, && X(0) = 0,\\
\dot{X}^* &= -\delta X^* + k_1 C_1 - a_2 X^*(M_T - C_2 - C_4) + d_2 C_2 - a_3 X^*(Z_T - C_1 - C_2) + d_3 C_3 + k_4 C_4, && X^*(0) = 0,\\
\dot{X}^{**} &= -\delta X^{**} + k_3 C_3 - a_4 X^{**}(M_T - C_2 - C_4) + d_4 C_4 - k_{\text{on}} X^{**} p_T(1 - c) + k_{\text{off}} c, && X^{**}(0) = 0,\\
\dot{C}_1 &= a_1 X(Z_T - C_1 - C_2) - (d_1 + k_1) C_1 - \delta C_1, && C_1(0) = 0,\\
\dot{C}_2 &= a_2 X^*(M_T - C_2 - C_4) - (d_2 + k_2) C_2 - \delta C_2, && C_2(0) = 0,\\
\dot{C}_3 &= a_3 X^*(Z_T - C_1 - C_2) - (d_3 + k_3) C_3 - \delta C_3, && C_3(0) = 0,\\
\dot{C}_4 &= a_4 X^{**}(M_T - C_2 - C_4) - (d_4 + k_4) C_4 - \delta C_4, && C_4(0) = 0,\\
\dot{C} &= k_{\text{on}} X^{**}(1 - c) - k_{\text{off}} c - \delta c, && c(0) = 0.
\end{aligned}
\tag{112}
$$

Based on the system of ODEs (112), we bring this system to form (1) as shown in Table 7. We now solve for the functions $\underline{\Psi}$ and ϕ as defined by Assumptions 5 and 6.

Solving for $\underline{X} = \underline{\Psi}$ by setting $(Br + f_1)_{6\times 1} = 0$, we have:

$$
\begin{aligned}
&(Br + f_1)_3 = 0 \implies a_1 X(Z_T - C_1 - C_3) = (d_1 + k_1 + \delta)\, C_1.\\
&\text{Under Assumption 1, } (d_1 + k_1) \gg \delta.\\
&\text{Thus, } X Z_T - X C_3 \approx (K_{m1} + X) C_1.\\
&\text{If } K_{m1} \gg X, \text{ we have: } X Z_T - X C_3 \approx K_{m1} C_1.\\
&(Br + f_1)_5 = 0 \implies a_3 X^*(Z_T - C_1 - C_3) = (d_3 + k_3 + \delta)\, C_3.\\
&\text{Under Assumption 1, } (d_3 + k_3) \gg \delta.\\
&\text{Thus, } X^* Z_T - X^* c_1 \approx (K_{m3} + X^*) C_3.\\
&\text{If } K_{m3} \gg X^*, \text{ we have: } X^* Z_T - X^* C_1 \approx K_{m3} C_3.
\end{aligned}
$$

Simultaneously solving these two expressions, for $K_{m1} \gg X$ and $K_{m3} \gg X^*$:

$$
\begin{aligned}
C_1 &\approx \frac{X Z_T}{K_{m1}},\\
C_3 &\approx \frac{X^* Z_T}{K_{m3}}.
\end{aligned}
\tag{113}
$$

U	X	v	c
$\underline{X}$	$\begin{bmatrix} X^* & X^{**} & C_1 & C_2 & C_3 & C_4 \end{bmatrix}^T_{6\times1}$	$Y,\ I$	$X^{**}, \begin{bmatrix} 0 & 1 & 0 & 0 & 0 & 0 \end{bmatrix}_{1\times6}$
G_1	$\max\left\{\frac{a_1Z_T}{\delta}, \frac{d_1}{\delta}, \frac{k_1}{\delta}, \frac{a_2M_T}{\delta}, \frac{d_2}{\delta}, \frac{k_2}{\delta}, \frac{a_3Z_T}{\delta}, \frac{d_3}{\delta}, \frac{k_3}{\delta}, \frac{a_4M_T}{\delta}, \frac{d_4}{\delta}, \frac{k_4}{\delta}\right\}$	G_2	$\max\left\{\frac{k_{\text{on}}p_T}{\delta}, \frac{k_{\text{off}}}{\delta}\right\}$
$f_0(U, R\underline{X}, S_1v, t)$	$k(t) - \delta(X + X^* + X^{**} + C_1 + C_2 + C_3 + C_4 + p_Tc)$	$s(\underline{X}, v)$	$\frac{1}{G_2}\left(k_{\text{on}}X^{**}(1-c) - k_{\text{off}}c - \delta c\right)$
$\underline{r}(U, \underline{X}, S_2v)$	$\frac{1}{G_1}\begin{bmatrix} \delta X^*, & \delta(X^{**} + p_Tc), & -a_1X(Z_T - C_1 - C_3) + d_1C_1 + \delta C_1, & k_2C_2 + \delta C_2, & \delta C_3, & \delta C_4 \end{bmatrix}^T_{6\times1}$		
$f_1(u, \underline{x}, S_3v)$	$\frac{1}{G_1}\begin{bmatrix} k_1C_1 - a_2X^*(M_T - C_2 - C_4) + d_2C_2 - a_3X^*(Z_T - C_1 - C_2) + d_3C_3 + k_4C_4, \\ k_3C_3 - a_4X^{**}(M_T - C_2 - C_4) + d_4C_4, \\ -k_1C_1, \\ a_2X^*(M_T - C_2 - C_4) - d_2C_2, \\ a_3X^*(Z_T - C_1 - C_2) - (d_3 + k_3)C_3, \\ a_4X^{**}(M_T - C_2 - C_4) - (d_4 + k_4)C_4 \end{bmatrix}_{6\times1}$		
A	$\begin{bmatrix} 1 & 1 & 1 & 1 & 1 & 1 \end{bmatrix}_{1\times6}$	D	1
B	$\begin{bmatrix} -1 & 0 & 0 & 0 & 0 & 0 \\ 0 & -1 & 0 & 0 & 0 & 0 \\ 0 & 0 & -1 & 0 & 0 & 0 \\ 0 & 0 & 0 & -1 & 0 & 0 \\ 0 & 0 & 0 & 0 & -1 & 0 \\ 0 & 0 & 0 & 0 & 0 & -1 \end{bmatrix}_{6\times6}$	C	$\begin{bmatrix} 0 \\ -p_T \\ 0 \\ 0 \\ 0 \\ 0 \end{bmatrix}_{6\times1}$
R	$\begin{bmatrix} 1 & 1 & 1 & 1 & 1 & 1 \end{bmatrix}_{1\times6}$	S_1	p_T
S_2	p_T	S_3	0
T	1	M	$\begin{bmatrix} 1 & 1 & 1 & 1 & 1 & 1 \end{bmatrix}_{1\times6}$
Q	$\mathbb{I}_{6\times6}$	P	$\begin{bmatrix} 0 & p_T & 0 & 0 & 0 & 0 \end{bmatrix}^T_{6\times1}$

Table 7. System variables, functions and matrices for a double phosphorylation cycle with substrate as input brought to form (1).

$(Br+f_1)_4=0 \implies a_2X^*(M_T-C_2-C_4)=(d_2+k_2+\delta)\,C_2.$
Under Assumption 1, $(d_2+k_2)\gg\delta$.
Thus, $X^*M_T-X^*C_4\approx(K_{m2}+X^*)C_2$.
If $K_{m2}\gg X^*: X^*M_T-X^*C_4\approx K_{m2}C_2$.
$(Br+f_1)_6=0 \implies a_4X^{**}(M_T-C_2-C_4)=(d_4+k_4+\delta)\,C_4$
Under Assumption 1, $(d_4+k_4)\gg\delta$.
Thus, $X^{**}M_T-X^{**}C_2=(K_{m4}+X^{**})C_4$.
If $K_{m4}\gg X^{**}, X^{**}M_T-X^{**}C_2\approx K_{m4}C_4$.

Simultaneously solving these two expressions, for $K_{m2}\gg X^*$ and $K_{m4}\gg X^{**}$:

$$C_2\approx\frac{X^*M_T}{K_{m2}},\qquad c_4\approx\frac{X^{**}M_T}{K_{m4}}. \tag{114}$$

$(Br+f_1)_2=0 \implies -\delta X^{**}-\delta p_Tc+k_3C_3-a_4X^{**}(M_T-C_2-C_4)+d_4C_4=0,$
using $(Br+f_1)_6=0, -\delta X^{**}-\delta p_Tc+k_3C_3-k_4c_4\approx 0.$

From (113) and (114), $-\delta X^{**}-\delta p_Tc+k_3X^*-k_4X^{**}\approx 0,$

$$\text{i.e., } X^{**}\approx\left(\frac{\frac{k_3Z_T}{K_{m3}}}{\delta+\frac{k_4M_T}{K_{m4}}}\right)X^*-\left(\frac{\delta p_T}{\delta+\frac{k_4M_T}{K_{m4}}}\right)c \tag{115}$$

$$X^{**}\approx K''X^*-K_c'c,\ \text{where } K''=\left(\frac{\frac{k_3Z_T}{K_{m3}}}{\delta+\frac{k_4M_T}{K_{m4}}}\right), K_c'=\left(\frac{\delta p_T}{\delta+\frac{k_4M_T}{K_{m4}}}\right).$$

$(Br+f_1)_1=0 \implies$
$-\delta X^*+k_1C_1-a_2X^*(M_T-C_2-C_4)+d_2C_2-a_3X^*(Z_T-C_1-C_3)+d_3C_3+k_4C_4=0,$
using $(Br+f_1)_4=0$ and $(Br+f_1)_5=0, -\delta X^*+k_1C_1-k_2C_2-k_3C_3+k_4C_4\approx 0.$

From (113), (114) and (115), $-\delta X^*+k_1X-k_2X^*-k_3X^*+k_4(K''X-K_c'c)X^*\approx 0,$
i.e., $X^*=K'X-K_c''c,$

$$\text{where } K'=\left(\frac{\frac{k_1Z_T}{K_{m1}}}{\delta+\frac{k_2M_T}{K_{m2}}+\frac{k_3Z_T}{K_{m3}}-K''\frac{k_4M_T}{K_{m4}}}\right)\text{ and } K_c''=\left(\frac{K_c'\frac{k_4M_T}{K_{m4}}}{\delta+\frac{k_2M_T}{K_{m2}}+\frac{k_3Z_T}{K_{m3}}-K''\frac{k_4M_T}{K_{m4}}}\right). \tag{116}$$

Thus, from equations (113)-(116), for K', K'', K_c' and K_c'' defined in (115) and (116), we have the function $\underline{\Psi}(U,v)$:

$$\underline{\Psi}\approx\begin{bmatrix} K'X-K_c''c,\\ K'K''x-(K''K_c''+K_c')c,\\ \frac{XZ_T}{K_{m1}},\\ \frac{1}{K_{m2}}(G'X-G_c''c),\\ \frac{X_T}{K_{m3}}(G'X-G_c''c),\\ \frac{1}{K_{m4}}(G'G''X-(G''G_c''+G_c')c)\end{bmatrix}_{6\times 1}. \tag{117}$$

Solving for ϕ by setting $s(\underline{X},v)=0$, we have:

$$\begin{aligned} k_{\text{on}}X^{**}(1-c)&=k_{\text{off}}c,\\ \text{i.e., } X^{**}-X^{**}c&=k_Dc,\\ \text{i.e., } \phi=c&=\frac{X^{**}}{k_D+X^{**}}.\end{aligned} \tag{118}$$

Here again, we find $\underline{\Gamma}$ from (117) and (118) under Remark 1, and find that it satisfies Assumption 7. We then state without proof the following claims for this system:

Claim 7. *For the matrix B and functions r, f_1 and s defined in Table 7, Assumption 3 is satisfied for this system.*

Claim 8. *For the functions f_0 and $\underline{r}$ and matrices R, S_1 and A defined in Table 7, and the functions $\underline{\gamma}$ and ϕ as found above, Assumption 9 is satisfied for this system.*

For matrices T, Q, M, P defined in Table 7, we see that Assumption 4 is satisfied. Further, for $\underline{\Psi}$ and ϕ defined by (117) and (118), Assumption 5 and 6 are satisfied. Thus, Theorems 1, 2 and 3 can be applied to this system.

Results: (i) Retroactivity to the input: From Table 7, we see that R and S_1 cannot be made small. Thus, under Theorem 1, h_1 and h_2 cannot be made small, and thus, retroactivity to the input cannot be made small.

(ii) Retroactivity to the output: From Table 7, S_1 and S_2 cannot be made small. Thus, under Theorem 2, $\bar{h}_1$ and h_2 cannot be made small, and thus, retroactivity to the output cannot be made small.

(iii) Input-output relationship: From (117),

$$Y_{is}(t) \approx I\underline{\Psi}(U_{is}, 0) = KX(t) \tag{119}$$

for $t \in [t_b, t_f]$. Thus the input-output relationship has $m = 1$ and $K = K'K''$ as defined in (115), (116), which can be tuned by tuning the total kinase and phosphatase concentrations Z_T and M_T.

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