Retroactivity Attenuation in Signaling Cascades

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Abstract— It has been shown in an earlier work that impedance-like effects, called retroactivity, are found at the interconnection of biomolecular systems just as they occur in several engineering systems. These effects are particularly relevant in signaling cascades that have several downstream targets. These cascades have been extensively studied to determine how a stimulus at the top of the cascade is transmitted and amplified as it propagates toward the bottom of the cascade. In principle, because of retroactivity, a perturbation at the bottom of the cascade can propagate upstream. In this paper, we study the extent to which this propagation occurs by analytically finding retroactivity gains at each stage of the cascade. These gains determine whether a perturbation at the bottom of the cascade is amplified or attenuated as it propagates upstream.

I. INTRODUCTION

A living cell is an intricate system that is composed of networks of molecules that transmit signals both within the cell, and between the cell and its environment. An important part of these networks consists of signaling pathways, which cover a central role in a cell's ability to sense and respond to both external and internal input stimuli [1], [2]. Often, signaling pathways consist of phosphorylation/dephosphorylation (PD) cycles, wherein a protein is reversibly converted between an active and an inactive form [3]. Several PD cycles often appear connected in a cascade fashion and the length of the cascade has been shown to have prominent effects, for instance, on signal amplification, signal duration, and signaling time [4]–[7]. Specifically, a wealth of work has been employing metabolic control analysis (MCA) to determine the gains across the cascade as a small amplitude stimulus is applied at the top of the cascade [7]–[9]. No study has been carried on how and whether perturbations at the bottom of a cascade propagate toward the top.

Cascades often intersect with each other through common components, such as protein substrates. Hence, perturbations at bottom or intermediate stages in a cascade are common and often lead to unwanted crosstalk between the signaling stages downstream of the intersection point [10]–[11]. No attention has been given to crosstalk between the stages *upstream* of the intersection point. In fact, several of these works, modeled a cascade as the modular composition of PD cycles, that is, unidirectional signal propagation was implicitly assumed. Theoretical work, however, has shown that biomolecular systems, among which PD cycles, cannot always be modularly connected with each other because of

retroactivity [12]–[15]. Preliminary experimental validation of the steady state effects of retroactivity have also appeared [16]–[18]. Retroactivity changes the behavior of an upstream system upon interconnection to downstream clients. This is especially relevant in signaling cascades, in which each PD cycle has several targets. As a result of retroactivity, signaling cascades allow bidirectional signal propagation, wherein a perturbation at the bottom of the cascade can propagate toward the top.

A perturbation at the bottom of the cascade can be due, for example, to a downstream protein substrate that is shared with another pathway so that the amount of target/substrate available to the cascade under study can change. Also, the introduction of an inhibitor, as in targeted drug design [19], creates a perturbation at a targeted stage. In this work, we quantify the effect of such perturbations on the upstream stages and investigate how the effects of retroactivity propagate upstream. On the one hand, this will reveal the extent by which aberrant signaling in the upstream stages of a cascade, as found in diseases such as cancer [20], can be caused by retroactivity. On the other hand, it will provide tools for targeted drug design by quantifying possible off-target effects of inhibitors.

From a synthetic biology perspective, modular design involves creating biological circuits that behave in isolation the same as when they are connected with other circuits. Therefore, by understanding the processes that attenuate the effects of retroactivity [15], [21], the circuit designer can design signaling pathways that amplify signals from upstream to downstream and attenuate signals from downstream to upstream, thereby enforcing unidirectional signal propagation which is crucial in modular design.

In this paper, we consider a PD cascade with a single phosphorylation cycle per stage and no explicit feedback, and we incorporate retroactivity explicitly in the PD cycle model. Our earlier work, [22], considers a similar problem with the difference that [22] employs a more complicated and complete model of such cascades. Here, we show that with a simpler model, qualitatively similar results are obtained. Specifically, we consider small perturbations at the bottom of the cascade and explicitly quantify how such perturbations propagate from downstream to upstream at steady state. Our main results are as follows. We provide analytical expressions for the downstream-to-upstream transmission gains. These establish the extent to which a perturbation at the bottom of the cascade can propagate upstream. We then provide several sufficient conditions for retroactivity attenuation. Furthermore, we highlight some important structural properties of these cascades. The paper
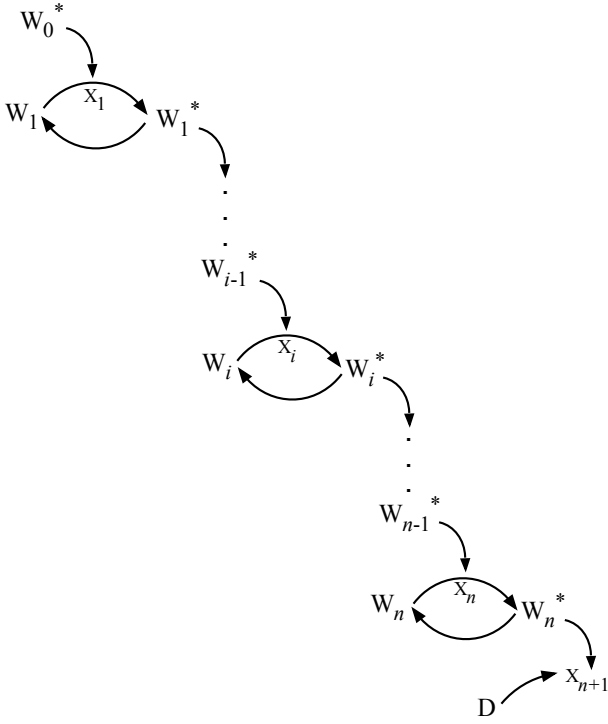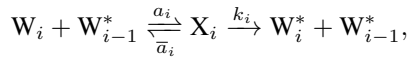
Fig. 1. A Phosphorylation/Dephosphorylation (PD) cascade with n stages.

is organized as follows: in Section II, we explain the cascade model. In Section III, we present our results. In Section IV, we demonstrate our results using numerical simulations. In Section V, we conclude with discussions and future work.

II. SYSTEM MODEL

Consider a signaling cascade comprised of n PD cycles as depicted in Fig. 1. At each stage i, $i \in \{1, \ldots, n\}$, W_{i-1}^* denotes the kinase, E_i denotes the phosphatase, W_i denotes the protein substrate, and W_i^* denotes the phosphorylated form of W_i. The kinase W_{i-1}^* binds to W_i to form the substrate-kinase complex X_i. This complex is then converted to W_i^*. The phosphorylated protein W_i^* is itself a kinase for the next downstream stage and, by binding to downstream substrates, forms the complex X_{i+1}. The phosphatase E_i activates the dephosphorylation of protein W_i^* and converts W_i^* to W_i. The phosphorylation reaction is given by

$$W_i + W_{i-1}^* \underset{\overline{a}_i}{\overset{a_i}{\rightleftharpoons}} X_i \overset{k_i}{\longrightarrow} W_i^* + W_{i-1}^*,$$

where $\overline{a}_i$, a_i, and k_i denote rates of reaction.

In this paper, we assume that the dephosphorylation reaction occurs in one step and no intermediate complex is formed. This is a good assumption if the catalytic rate of reaction for dephosphorylation is sufficiently fast. The dephosphorylation reaction is, thus, given by

$$W_i^* \overset{b_i E_{iT}}{\longrightarrow} W_i,$$
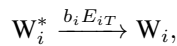
where E_{iT} is the total amount of phosphatase in stage i and b_i is the rate at which W_i^* binds to the phosphatase. We

assume that protein W_i is conserved at every stage and is in total amount W_{iT}. Therefore, we have that

$$W_i + W_i^* + X_i + X_{i+1} = W_{iT}, \tag{1}$$

in which for a species X, X (italics) denotes its concentration. We assume that the kinase to the first stage, W_0^*, is produced at rate $k(t)$ and decays at rate δ, i.e.,

$$W_0^* \underset{k(t)}{\overset{\delta}{\rightleftharpoons}} \emptyset.$$

Finally, we assume that the phosphorylated protein of the last stage, W_n^*, binds to species D downstream of the cascade and forms the complex X_{n+1}, that is,

$$W_n^* + D \underset{\overline{a}_{n+1}}{\overset{a_{n+1}}{\rightleftharpoons}} X_{n+1} \text{ with } D_T := D + X_{n+1}.$$
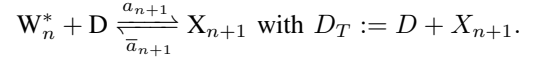
Species D could model, for instance, a substrate that is shared with other signaling pathways. It could also model a signaling molecule or an inhibitor of the active enzyme W_n^*, as considered in targeted drug design, in which the total concentration of D could be perturbed by the addition of more drug. Therefore, the amount of free D plus the amount of D bound to W_n^*, which we call D_T, could be perturbed. In this study, we consider a perturbation in D_T as the *input* to the system. Specifically, we assume a small perturbation on D_T and calculate the sensitivity of the steady state response of W_i^*, $i \in \{1, \ldots, n\}$.

A standing assumption in this paper is that the length of the cascade, n, is finite, and for all $i \in \{1, \ldots, n\}$, the quantities W_{iT}, E_{iT}, a_i, $\overline{a}_i$, k_i, b_i, δ, a_{n+1}, and $\overline{a}_{n+1}$ are finite and strictly positive.

Based on the given chemical reactions, the differential equations that describe the dynamics of the system are

$$\dot{W}_0^* = -\delta W_0^* + k(t) - \boxed{(a_1 W_0^* W_1 - (\overline{a}_1 + k_1) X_1)}$$
$$\dot{X}_i = a_i W_{i-1}^* W_i - (\overline{a}_i + k_i) X_i$$
$$\dot{W}_i^* = k_i X_i - b_i E_{iT} W_i^* -$$
$$\boxed{(a_{i+1} W_i^* W_{i+1} - (\overline{a}_{i+1} + k_{i+1}) X_{i+1})}$$
$$\dot{W}_n^* = k_n X_n - b_n E_{nT} W_n^* -$$
$$\boxed{(a_{n+1} D W_n^* - \overline{a}_{n+1} X_{n+1})}$$
$$\dot{X}_{n+1} = a_{n+1}(D_T - X_{n+1}) W_n^* - \overline{a}_{n+1} X_{n+1},$$

where $i \in \{1, \ldots, n\}$. Noting that the terms in the boxes correspond to $\dot{X}_1$, $\dot{X}_{i+1}$, and $\dot{X}_{n+1}$, respectively, and employing the conservation law (1), we obtain, for $i \in \{1, \ldots, n\}$, that

$$\dot{W}_0^* = -\delta W_0^* + k(t) - \dot{X}_1$$
$$\dot{X}_i = a_i W_{i-1}^*(W_{iT} - W_i^* - X_i - X_{i+1}) -$$
$$(\overline{a}_i + k_i) X_i \tag{2}$$
$$\dot{W}_i^* = k_i X_i - b_i E_{iT} W_i^* - \dot{X}_{i+1}$$
$$\dot{X}_{n+1} = a_{n+1}(D_T - X_{n+1}) W_n^* - \overline{a}_{n+1} X_{n+1}.$$

III. RESULTS

In this section, we assume that the cascade is at the steady state and explore how a small perturbation in the concentration D_T perturbs the steady state concentrations of each stage of the cascade. Let $0 < \overline{k} < \infty$ and $0 < \overline{D}_T < \infty$ denote the steady state values of $k(t)$ and D_T, respectively. Let the corresponding equilibrium values of W_0^*, W_i^*, X_i, W_i, for $i \in \{1, \cdots, n\}$, and X_{n+1} be denoted by $\overline{W}_0^*$, $\overline{W}_i^*$, $\overline{X}_i$, $\overline{W}_i$, for $i \in \{1, \cdots, n\}$, and $\overline{X}_{n+1}$, respectively. Let $d_T := D_T - \overline{D}_T$ represent the perturbation of D_T with respect to its steady state value. The corresponding perturbations of the states of the cascade about the equilibrium values are denoted by w_0^*, w_i^*, x_i for $i \in \{1, \cdots, n\}$, and x_{n+1}. Similarly, let Z_i, for $i \in \{1, \ldots, n\}$, denote the concentration of the *total* phosphorylated protein at stage i, i.e., $Z_i := W_i^* + X_{i+1}$. This concentration is easy to measure experimentally in the lab and will thus be also studied here. Let the corresponding perturbation about the steady state $\overline{Z}_i = \overline{W}_i^* + \overline{X}_{i+1}$ be denoted by z_i, which can be written as $z_i = w_i^* + x_{i+1}$ for all $i \in \{1, \ldots, n\}$. To quantify the effects of retroactivity, we introduce the following gains, for $i \in \{1, \ldots, n-1\}$:

$$\Psi_i = \left| \frac{w_i^*}{w_{i+1}^*} \right|, \quad \Phi_i = \left| \frac{z_i}{z_{i+1}} \right|. \tag{3}$$

Definition 1. We say that stage i attenuates retroactivity with respect to the *free* phosphorylated protein provided $\Psi_i < 1$.

Definition 2. We say that stage i attenuates retroactivity with respect to the *total* phosphorylated protein provided $\Phi_i < 1$.

To understand how retroactivity propagates from each stage to the next stage upstream, we quantify Ψ_i and Φ_i, for $i \in \{1, \ldots, n-1\}$ as functions of the cascade parameters. To achieve this, we assume that d_T, viewed as the input to the system, is sufficiently small, and we linearize the dynamics of system (2) about the equilibrium as follows:

$$
\begin{aligned}
\dot{w}_0^* &= -\delta w_0^* - \dot{x}_1 \\
\dot{x}_i &= a_i \overline{W}_{i-1}^*(-w_i^* - x_i - x_{i+1}) + a_i \overline{W}_i w_{i-1}^* - \\
&\quad (\overline{a_i} + k_i)x_i \\
\dot{w}_i^* &= k_i x_i - b_i E_{iT} w_i^* - \dot{x}_{i+1} \\
\dot{x}_{n+1} &= a_{n+1} \overline{D} w_n^* + a_{n+1} \overline{W}_n^*(d_T - x_{n+1}) - \\
&\quad \overline{a}_{n+1} x_{n+1},
\end{aligned}
\tag{4}
$$

in which we have, for $i \in \{1, \cdots, n\}$, that (from setting the time derivatives in equations (2) to zero)

$$\overline{W}_0^* = \frac{\overline{k}}{\delta}, \quad \overline{W}_i = \alpha_i \frac{b_i}{k_i} E_{iT} \frac{\overline{W}_i^*}{\overline{W}_{i-1}^*}, \tag{5}$$

where $\alpha_i := \frac{\overline{a}_i + k_i}{a_i}$ is the Michaelis-Menten constant of the phosphorylation reaction. Since we are interested in the steady state of the linearized model, we set the time derivatives in system (4) to zero and simplify to obtain
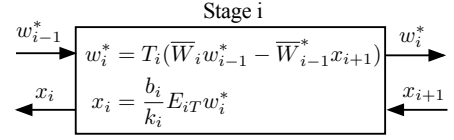
Fig. 2. Directionality of signals in stage i. Retroactivity to the output of stage i is due to the perturbation of the complex X_{i+1}.

$$w_0^* = 0 \tag{6}$$

$$x_i = \frac{b_i}{k_i} E_{iT} w_i^* \tag{7}$$

$$w_i^* = T_i(\overline{W}_i w_{i-1}^* - \overline{W}_{i-1}^* x_{i+1}) \tag{8}$$

$$x_{n+1} = \frac{\overline{D} w_n^* + \overline{W}_n^* d_T}{\overline{W}_n^* + \frac{\overline{a}_{n+1}}{a_{n+1}}}, \tag{9}$$

where

$$T_i = \frac{1}{\overline{W}_{i-1}^* + \frac{b_i}{k_i} E_{iT} \left(\overline{W}_{i-1}^* + \alpha_i \right)}. \tag{10}$$

Fig. 2 represents equations (7) and (8) as a block diagram, which highlights the directionality of signal propagation through each stage of the cascade. Basically, perturbation d_T causes perturbation x_{n+1}, which then propagates to upstream stages in the cascade through perturbations x_i. Hence, in this steady state response model, retroactivity is due to perturbations in the concentration of the complexes X_i. We now provide some structural properties of PD cascades.

Lemma 1: The n-stage cascade defined by (6)–(9) is such that $sign(w_i^*) = -sign(w_{i+1}^*)$ for all $i \in \{1, ..., n-1\}$.

Proof: (By induction on the stage number i)
Base case: For $i = 1$, (6) − (8) yield

$$w_1^* = -\left(T_1 \overline{W}_0^* \frac{b_2}{k_2} E_{2T} \right) w_2^*. \tag{11}$$

Since $T_1 \overline{W}_0^* \frac{b_2}{k_2} E_{2T} \geq 0$, (11) shows that $sign(w_1^*) = -sign(w_2^*)$.

Induction Step: Assume that $sign(w_{i-1}^*) = -sign(w_i^*)$. We prove that $sign(w_i^*) = -sign(w_{i+1}^*)$. Employing equations (7) and (8), we have that

$$w_i^* = T_i \overline{W}_i w_{i-1}^* - T_i \overline{W}_{i-1}^* \frac{b_{i+1}}{k_{i+1}} E_{(i+1)T} w_{i+1}^*.$$

To simplify notation, define $G_1 := T_i \overline{W}_i$ and $G_2 := T_i \overline{W}_{i-1}^* \frac{b_{i+1}}{k_{i+1}} E_{(i+1)T}$. Then, we have that $w_i^* = G_1 w_{i-1}^* - G_2 w_{i+1}^*$ from which we obtain that

$$w_{i+1}^* = \frac{G_1}{G_2} w_{i-1}^* - \frac{1}{G_2} w_i^*. \tag{12}$$

In order to proceed, we consider two cases and employ the fact that $G_1 \geq 0$ and $G_2 \geq 0$.

case 1: If $w_{i-1}^* \geq 0$, then by the induction assumption $w_i^* \leq 0$; hence by (12) we obtain that

$$w_{i+1}^* = \frac{G_1}{G_2} |w_{i-1}^*| + \frac{1}{G_2} |w_i^*| \geq 0.$$

case 2: If $w_{i-1}^* \leq 0$, then by the induction assumption $w_i^* \geq 0$; hence by (12) we obtain that

$$w_{i+1}^* = -\frac{G_1}{G_2}|w_{i-1}^*| - \frac{1}{G_2}|w_i^*| \leq 0.$$

This proves the lemma. ∎

This lemma implies that if the downstream perturbation d_T causes a decrease in the free phosphorylated protein concentration at one stage, it causes an increase in that of the next upstream stage. We now quantify Ψ_i defined in (3):

Theorem 1: The stage gain Ψ_i, defined in (3), is given by

$$\Psi_i = \frac{\frac{b_{i+1}}{k_{i+1}}E_{(i+1)T}}{1 + \frac{b_i}{k_i}E_{iT}\left(1 + \frac{\alpha_i}{\overline{W}_{i-1}^*}\left(1 + \frac{\overline{W}_i^*}{\overline{W}_{i-1}^*}\Psi_{i-1}\right)\right)} \quad (13)$$

with $\Psi_0 := 0$.

Proof: (By induction on stage number i)

Base case: From equations (6) – (8), we have that $w_1^* = -T_1 \overline{W}_0^* \frac{b_2}{k_2} E_{2T} w_2^*$ from which we obtain that $|w_1^*| = T_1 \overline{W}_0^* \frac{b_2}{k_2} E_{2T} |w_2^*|$. Substituting for T_1 from equation (10) and simplifying gives

$$|w_1^*| = \frac{\frac{b_2}{k_2}E_{2T}}{1 + \frac{b_1}{k_1}E_{1T}\left(1 + \frac{\alpha_1}{\overline{W}_0^*}\right)}|w_2^*|.$$

Thus $\Psi_1 = \dfrac{\frac{b_2}{k_2}E_{2T}}{1 + \frac{b_1}{k_1}E_{1T}\left(1 + \frac{\alpha_1}{\overline{W}_0^*}\right)}$ and the base case is proven.

Induction step: Assume $|w_{i-1}^*| = \Psi_{i-1}|w_i^*|$. We prove that $|w_i^*| = \Psi_i|w_{i+1}^*|$. By Lemma 1, $sign(w_i^*) = -sign(w_{i-1}^*)$. Therefore, the induction assumption becomes $w_{i-1}^* = -\Psi_{i-1}w_i^*$. Employing equations (7) and (8) as well as the induction assumption, we obtain that

$$w_i^* = -T_i\overline{W}_i\Psi_{i-1}w_i^* - T_i\overline{W}_{i-1}^*\frac{b_{i+1}}{k_{i+1}}E_{(i+1)T}w_{i+1}^*.$$

Substituting for $\overline{W}_i$ from equation (5) and simplifying we obtain that

$$w_i^* = -\frac{\frac{b_{i+1}}{k_{i+1}}E_{(i+1)T}}{\frac{1}{T_i\overline{W}_{i-1}^*} + \frac{\overline{W}_i}{\overline{W}_{i-1}^*}\Psi_{i-1}}w_{i+1}^*$$

$$= -\frac{\frac{b_{i+1}E_{(i+1)T}}{k_{i+1}}w_{i+1}^*}{1 + \frac{b_iE_{iT}}{k_i}(1 + \frac{\alpha_i}{\overline{W}_{i-1}^*}) + \alpha_i\frac{b_iE_{iT}}{k_i}\frac{\overline{W}_i^*}{\overline{W}_{i-1}^*}\frac{1}{\overline{W}_{i-1}^*}\Psi_{i-1}}$$

$$= -\frac{\frac{b_{i+1}E_{(i+1)T}}{k_{i+1}}}{1 + \frac{b_iE_{iT}}{k_i}\left(1 + \frac{\alpha_i}{\overline{W}_{i-1}^*}(1 + \frac{\overline{W}_i^*}{\overline{W}_{i-1}^*}\Psi_{i-1})\right)}w_{i+1}^*.$$

Since $\dfrac{\frac{b_{i+1}E_{(i+1)T}}{k_{i+1}}}{1 + \frac{b_iE_{iT}}{k_i}\left(1 + \frac{\alpha_i}{\overline{W}_{i-1}^*}(1 + \frac{\overline{W}_i^*}{\overline{W}_{i-1}^*}\Psi_{i-1})\right)} = \Psi_i$, with Ψ_i as defined in (13), $|w_i^*| = \Psi_i|w_{i+1}^*|$. ∎

The expressions of the gains Ψ_i are recursive. The following proposition provides non-recursive sufficient conditions for retroactivity attenuation.

Proposition 1: Stage i attenuates retroactivity with respect to the free phosphorylated protein if any of the following conditions are satisfied:

$$\frac{\frac{b_{i+1}E_{(i+1)T}}{k_{i+1}}}{1 + \frac{b_iE_{iT}}{k_i}(1 + \frac{\alpha_i}{\overline{W}_{i-1}^*})} < 1; \quad (14)$$

$$\frac{\frac{b_{i+1}E_{(i+1)T}}{k_{i+1}}}{1 + \frac{b_iE_{iT}}{k_i}(1 + \frac{\alpha_i}{W_{(i-1)T}})} < 1; \quad (15)$$

$$\frac{b_{i+1}E_{(i+1)T}}{k_{i+1}} < 1. \quad (16)$$

Proof: Since all terms in (13) are strictly positive, and that $\overline{W}_{iT}^* < W_{iT}$ from conservation law (1), it follows that Ψ_i is less than each of the expressions in the left hand side of (14)–(16). Therefore, if any of (14)–(16) hold, then $\Psi_i < 1$ and stage i attenuates retroactivity with respect to the free phosphorylated protein. ∎

From (15), it follows that stage i attenuates retroactivity if $W_{(i-1)T} \ll 1$, i.e., sufficiently small total kinase for stage i, and/or $\alpha_i \gg 1$, i.e., sufficiently large Michaelis-Menten constant for phosphorylation in stage i.

In the case of a weakly activated pathway [5], an interesting conclusion can be made. In such pathways $\overline{W}_{i-1}^* \ll W_{(i-1)T}$. Assuming, in addition, that $\overline{W}_{i-1}^* \ll \alpha_i$, left hand side of (14) can be approximated by

$$\left(\frac{\frac{b_{i+1}E_{(i+1)T}}{k_{i+1}}}{\frac{b_iE_{iT}}{k_i}}\right)\left(\frac{\overline{W}_{i-1}^*}{\alpha_i}\right).$$

If the term inside the first parentheses is not large (i.e., the rate of dephosphorylation compared to the catalytic rate of phosphorylation in stage $i + 1$ is not much larger than that of stage i), then $\Psi_i \ll 1$ and stage i is a good retroactivity attenuator.

In [5], it was shown that for weakly activated pathways, in order to have *upstream to downstream* signal amplification, it is necessary that the phosphorylation rate constant be larger than the dephosphorylation rate constant. For a weakly activated pathway in which $W_{(i-1)T} \ll \alpha_i$, the phosphorylation rate constant is well approximated by k_iW_{iT}/α_i [22]. Also, in our model, the dephosphorylation rate constant is b_iE_{iT}. Consequently, to have upstream-to-downstream signal amplification, it is required that

$$\frac{b_iE_{iT}}{k_iW_{iT}/\alpha_i} < 1,$$

which, when $\alpha_i \geq W_{iT}$, implies that $b_iE_{iT}/k_i < 1$. Based on the sufficient condition given in (16), this implies that $\Psi_{i-1} < 1$. Hence, the downstream perturbation is attenuated as it propagates from stage i to stage $i - 1$. Concluding, in weakly activated pathways in which $W_{iT} \leq \alpha_i$ and $W_{(i-1)T} \ll \alpha_i$, upstream to downstream signal amplification is associated with retroactivity attenuation. This, in turn, implies *unidirectional* signal propagation from upstream to downstream.

We now describe two other important structural properties of the cascade:

Theorem 2: The n-stage signaling cascade defined by (6)–(9) is such that $sign(z_i) = -sign(z_{i+1})$ for all $i \in \{1, ..., n-1\}$. Furthermore, Φ_i, defined in (3), is such that $\Phi_i < 1$, $i \in \{1, \ldots, n-1\}$.

Proof: To simplify notation, define for all $i \in \{1, \ldots, n\}$,

$$F_i := \frac{b_i E_{iT}}{k_i}\left(1 + \frac{\alpha_i}{\overline{W}_{i-1}^*}(1 + \frac{\overline{W}_i^*}{\overline{W}_{i-1}^*}\Psi_{i-1})\right), \quad (17)$$

so that Ψ_i can be written as

$$\Psi_i = \frac{1}{1 + F_i}\frac{b_{i+1}E_{(i+1)T}}{k_{i+1}}. \quad (18)$$

Now, substituting $w_{i-1}^* = -\Psi_{i-1}w_i^*$ in (8) yields

$$w_i^* = T_i(-\overline{W}_i\Psi_{i-1}w_i^* - \overline{W}_{i-1}x_{i+1}).$$

Substituting for $\overline{W}_i$ from (5) and solving for w_i^* yields

$$w_i^* = -\frac{x_{i+1}}{\frac{1}{T_i\overline{W}_{i-1}} + \frac{\overline{W}_i}{\overline{W}_{i-1}^*}\Psi_{i-1}} = -\frac{x_{i+1}}{1 + F_i},$$

where F_i is given by (17). Equivalently, $x_{i+1} = -(1+F_i)w_i^*$. We now express z_i in terms of w_i^* as follows:

$$z_i = w_i^* + x_{i+1} = w_i^* - (1+F_i)w_i^* = -F_i w_i^*. \quad (19)$$

Since $F_i > 0$, we obtain from (19) that $sign(z_i) = -sign(w_i^*)$ and, employing Lemma 1, we obtain that $sign(z_i) = -sign(z_{i+1})$. This proves the first part of the theorem. To prove the second part, we employ (19) in (3):

$$\Phi_i = \left|\frac{z_i}{z_{i+1}}\right| = \left|\frac{-F_i w_i^*}{-F_{i+1}w_{i+1}^*}\right| = \frac{F_i}{F_{i+1}}\left|\frac{w_i^*}{w_{i+1}^*}\right| = \frac{F_i}{F_{i+1}}\Psi_i.$$

Substituting for Ψ_i from (18), we obtain that

$$\Phi_i = \frac{F_i}{F_{i+1}}\frac{\frac{b_{i+1}E_{(i+1)T}}{k_{i+1}}}{1 + F_i} = \frac{F_i}{1 + F_i}\frac{\frac{b_{i+1}E_{(i+1)T}}{k_{i+1}}}{F_{i+1}}.$$

Since $F_i > 0$, we have that $\frac{F_i}{1+F_i} < 1$. Furthermore,

$$\frac{\frac{b_{i+1}E_{(i+1)T}}{k_{i+1}}}{F_{i+1}} = \frac{\frac{b_{i+1}E_{(i+1)T}}{k_{i+1}}}{\frac{b_{i+1}E_{(i+1)T}}{k_{i+1}}\left(1 + \frac{\alpha_{i+1}}{\overline{W}_i^*}(1 + \frac{\overline{W}_{i+1}^*}{\overline{W}_i^*}\Psi_i)\right)}$$

$$= \frac{\frac{b_{i+1}E_{(i+1)T}}{k_{i+1}}}{\frac{b_{i+1}E_{(i+1)T}}{k_{i+1}} + \frac{b_{i+1}E_{(i+1)T}}{k_{i+1}}\frac{\alpha_{i+1}}{\overline{W}_i^*}(1 + \frac{\overline{W}_{i+1}^*}{\overline{W}_i^*}\Psi_i)} < 1,$$

since all the terms are strictly positive. Thus, $\Phi_i < 1$. ∎

The first part of this theorem states that, similar to the case of free phosphorylated protein as described in Lemma 1, an increase in Z_{i+1} implies a decrease in Z_i. The second part of this theorem states that the magnitude of the perturbation at every stage is *always* attenuated as it propagates upstream in the cascade, regardless of the cascade parameters or the length of the cascade. In other words, every stage attenuates retroactivity with respect to the total phosphorylated protein. We now investigate how d_T affects w_n^* and z_n.

Theorem 3: The n-stage signaling cascade defined by (6)–(9) is such that $|w_n^*| < |d_T|$ and $|z_n| < |d_T|$.

Proof: To prove that $|w_n^*| < |d_T|$, we substitute x_{n+1} from (9) into (8) (with $i = n$) and use the fact that $w_{n-1}^* = -\Psi_{n-1}w_n^*$ to obtain

$$w_n^* = T_n(-\overline{W}_n\Psi_{n-1}w_n^* - \overline{W}_{n-1}^*\frac{\overline{D}w_n^* + \overline{W}_n^* d_T}{\overline{W}_n^* + \frac{\overline{a}_{n+1}}{a_{n+1}}}). \quad (20)$$

To simplify notation, let

$$K_1 := \frac{1}{1 + T_n(\overline{W}_n\Psi_{n-1} + \overline{W}_{n-1}^*\frac{1}{\overline{W}_n^* + \frac{\overline{a}_{n+1}}{a_{n+1}}}\overline{D})} \quad (21)$$

$$K_2 := T_n\overline{W}_{n-1}^* = \frac{1}{1 + \frac{b_n E_{nT}}{k_n}(1 + \frac{\alpha_n}{\overline{W}_{n-1}^*})} \quad (22)$$

$$K_3 := \frac{\overline{W}_n^*}{\overline{W}_n^* + \frac{\overline{a}_{n+1}}{a_{n+1}}}. \quad (23)$$

Therefore, solving (20) for w_n^*, we have that $w_n^* = -K_1 K_2 K_3 d_T$, i.e., $|w_n^*| = K_1 K_2 K_3 |d_T|$. From (21)–(23), it follows that $0 < K_i < 1$ for $i \in \{1, 2, 3\}$. Therefore, $|w_n^*| < |d_T|$. This proves the first part of the theorem. To prove the second part, we employ $w_n^* = -K_1 K_2 K_3 d_T$ and equation (9) to express z_n in terms of d_T as follows:

$$z_n = w_n^* + x_{n+1} = w_n^* + \frac{1}{\overline{W}_n^* + \frac{\overline{a}_{n+1}}{a_{n+1}}}\left(\overline{D}w_n^* + \overline{W}_n^* d_T\right)$$

$$= (1 + \frac{\overline{D}}{\overline{W}_n^* + \frac{\overline{a}_{n+1}}{a_{n+1}}})w_n^* + \frac{\overline{W}_n^*}{\overline{W}_n^* + \frac{\overline{a}_{n+1}}{a_{n+1}}}d_T$$

$$= \left(-(1 + \frac{\overline{D}}{\overline{W}_n^* + \frac{\overline{a}_{n+1}}{a_{n+1}}})(K_1 K_2 K_3) + \frac{\overline{W}_n^*}{\overline{W}_n^* + \frac{\overline{a}_{n+1}}{a_{n+1}}}\right)d_T$$

$$= K_3\left(-(1 + \frac{\overline{D}}{\overline{W}_n^* + \frac{\overline{a}_{n+1}}{a_{n+1}}})(K_1 K_2) + 1\right)d_T.$$

Since we have already shown that $0 < K_3 < 1$, in order to prove that $|z_n| < |d_T|$, it suffices to show that

$$0 < (1 + \frac{\overline{D}}{\overline{W}_n^* + \frac{\overline{a}_{n+1}}{a_{n+1}}})(K_1 K_2) < 1.$$

Since every term in this expression is strictly positive, the left hand side inequality follows. To show the right hand side inequality, we expand K_1 and K_2 and simplify as follows:

$$(1 + \frac{\overline{D}}{\overline{W}_n^* + \frac{\overline{a}_{n+1}}{a_{n+1}}})(K_1 K_2) =$$

$$(1 + \frac{\overline{D}}{\overline{W}_n^* + \frac{\overline{a}_{n+1}}{a_{n+1}}})\frac{T_n\overline{W}_{n-1}^*}{1 + T_n(\overline{W}_n\Psi_{n-1} + \overline{W}_{n-1}^*\frac{1}{\overline{W}_n^* + \frac{\overline{a}_{n+1}}{a_{n+1}}}\overline{D})}$$

$$= \frac{1 + \frac{\overline{D}}{\overline{W}_n^* + \frac{\overline{a}_{n+1}}{a_{n+1}}}}{\frac{1}{T_n\overline{W}_{n-1}^*} + \frac{\overline{W}_n}{\overline{W}_{n-1}^*}\Psi_{n-1} + \frac{1}{\overline{W}_n^* + \frac{\overline{a}_{n+1}}{a_{n+1}}}\overline{D}}$$

$$= \frac{(1 + \frac{\overline{D}}{\overline{W}_n^* + \frac{\overline{a}_{n+1}}{a_{n+1}}})}{(1 + \frac{\overline{D}}{\overline{W}_n^* + \frac{\overline{a}_{n+1}}{a_{n+1}}}) + (\frac{b_n E_{nT}}{k_n}(1 + \frac{\alpha_n}{\overline{W}_{n-1}^*}) + \frac{\overline{W}_n}{\overline{W}_{n-1}^*}\Psi_{n-1})},$$

Parameter	Value
k_i, $i = 1, 2, 3$	150 $(\text{min})^{-1}$
a_i, $i = 1, 2, 3$	2.5 $(\text{nM min})^{-1}$
$\overline{a}_i$, $i = 1, 2, 3$	600 $(\text{min})^{-1}$
b_i, $i = 1, 2, 3$	2.5 $(\text{nM min})^{-1}$,
E_{3T}	120 nM
E_{2T}	0.3 nM
E_{1T}	0.3 nM
W_{3T}	1200 nM
W_{2T}	1200 nM
W_{1T}	3 nM
$\overline{W}_0^*$	0.3 nM
$\overline{D}_T$	0 nM.

TABLE I

PARAMETERS USED FOR THE NUMERICAL SIMULATIONS.

which, since all terms are strictly positive, shows that $(1 + \frac{\overline{D}}{\overline{W}_n^* + \frac{\overline{a}_{n+1}}{a_{n+1}}})(K_1 K_2) < 1$. Therefore, $|z_n| < |d_T|$. ∎

This theorem states that perturbation d_T induces perturbations w_n^* and z_n that are less than d_T in magnitude, regardless of cascade parameters or the size of the cascade. Therefore, to study the upstream propagation of d_T, it suffices to study Ψ_i and Φ_i, as performed in Theorems 1 and 2.

Theorems 2 and 3 imply that $|z_1| < \cdots < |z_n| < |d_T|$, i.e., retroactivity is attenuated at every stage of the cascade and also from the disturbance to the last stage. Hence, the more stages there are in the cascade, the more the overall retroactivity attenuation from the disturbance to the top of the cascade. This is an important structural property of such cascades as it does not depend on specific parameter values.

To summarize, the signaling cascade described by (6)–(9) is such that $sign(w_i^*) = -sign(w_{i+1}^*)$ and $sign(z_i) = -sign(z_{i+1})$, for all $i \in \{1, ..., n-1\}$. Furthermore, the effects of retroactivity are always attenuated with respect to the total phosphorylated protein. In the case of free phosphorylated protein, both attenuation and amplification of retroactivity are possible. However, attenuation takes place when $W_{(i-1)T} \ll 1$ and/or $\alpha_i \gg 1$. Finally, in a weakly activated pathway in which $W_{iT} \leq \alpha_i$ and $W_{(i-1)T} \ll \alpha_i$, if stage i attenuates retroactivity, then it amplifies the upstream signal, enforcing unidirectional signal transmission.

As a final remark, it should be noted that the linearization considered in this paper is valid only when the perturbation d_T is small enough. Using numerical simulations, presented in the next section and in [22], we have observed that the linear approximation is in fact valid, even for large perturbations (i.e., perturbations that are comparable in size to the total protein concentration W_{nT}).

IV. NUMERICAL SIMULATIONS

In this section, we demonstrate our results on a three-stage PD cascade. All simulations are performed on the full nonlinear model of Eq. (2) using the ODE23s solver in the MATLAB computational environment. The parameters used for simulations are taken from [23] and are provided in Table 1.

Assuming zero initial conditions, i.e., $W_i^*(0) = 0$ and $X_i(0) = 0$, $i = 1, 2, 3$, Fig. 3 shows the trajectories of W_1^*, W_2^*, and W_3^* as a function of time. We assume that the total concentration D_T is zero for $0 \leq t \leq 30$ minutes, and suddenly changes to 1200 nM at $t = 30$ minutes.

As this figure illustrates, after some transient, the perturbation in D_T results in steady state perturbations in W_1^*, W_2^*, and W_3^*. Furthermore, the perturbation in W_3^* and W_1^* are negative, while that in W_2^* is positive. This validates Lemma 1. Finally, the figure shows that the size of the perturbation decreases as the stage number decreases, which implies retroactivity attenuation between all stages.

Fig. 4 illustrates the relationship between the perturbation in D_T (denoted by d_T) and the perturbations in W_1^*, W_2^*, and W_3^* (denoted, respectively, by w_1^*, w_2^*, and w_3^*). Surprisingly, the relationship between w_i^* and d_T is approximately linear even for large perturbations (up to 1200 nM). Hence, the theoretical results of this paper must hold. Note also that the values of w_1^* and w_3^* are negative while that of w_2^* is positive. As before, this validates Lemma 1. The gains Ψ_i, which can be computed either from the slope of the lines in Fig. 4 or from (1), are given by $\Psi_1 = 8.33 \times 10^{-4}$ and $\Psi_2 = 0.3$. Since Ψ_1 and Ψ_2 are both smaller than 1, the cascade should attenuate the downstream perturbation at every stage. This is confirmed by Figures 3 and 4. Since the values of Ψ_i are much smaller than 1, this three-stage cascade practically enforces unidirectional signal propagation from upstream to downstream.

Finally, the values of the gains Φ_i are computed to be $\Phi_1 = 7.33 \times 10^{-4}$ and $\Phi_2 = 0.25$, which are both smaller than one. This validates Theorem 2.

V. CONCLUSIONS AND FUTURE WORKS

This paper presented a steady state model of PD cascades in which the phosphorylation reaction takes place in two steps while the dephosphorylation reaction occurs in one step. We considered a linearized model and investigated how a downstream perturbation propagates upstream in the cascade. In particular, we derived expressions for the gains between successive stages and explored conditions under which the gains are smaller than one, implying retroactivity attenuation. For the case of weakly activated pathways, it was found that under mild conditions, attenuation of retroactivity is associated with amplification of upstream signals. We described some important structural properties of the cascade such as sign reversal of perturbation at each stage and attenuation of retroactivity in the case of total phosphorylated protein. We also explained that with more stages, more attenuation in the total phosphorylated protein can be obtained. In [22], it was shown that longer cascades better attenuate retroactivity with respect to the *free* phosphorylated protein as well. Finally, we confirmed our results using numerical simulations which were based on biologically relevant parameters.

Future work includes extending the results to transient response. Transients could become significant if the pertur-
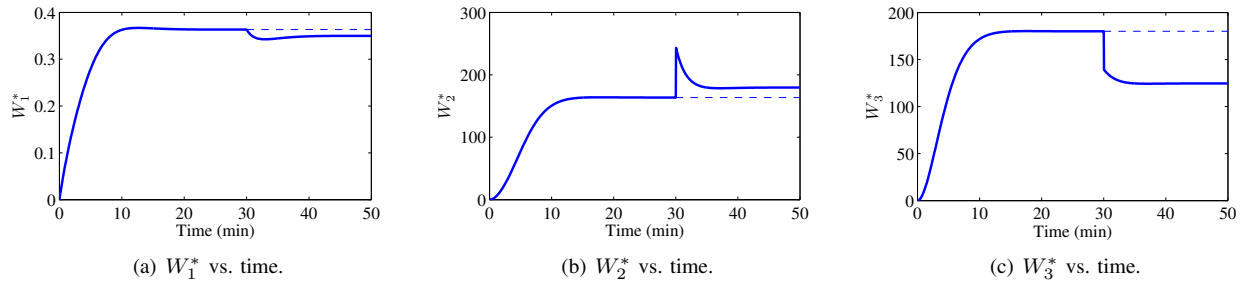
(a) W_1^* vs. time. (b) W_2^* vs. time. (c) W_3^* vs. time.

Fig. 3. Time response of the system to zero initial conditions and parameters given in Table 1. The concentration D_T changes suddenly from 0 to 1200 nM at $t = 30$ min.
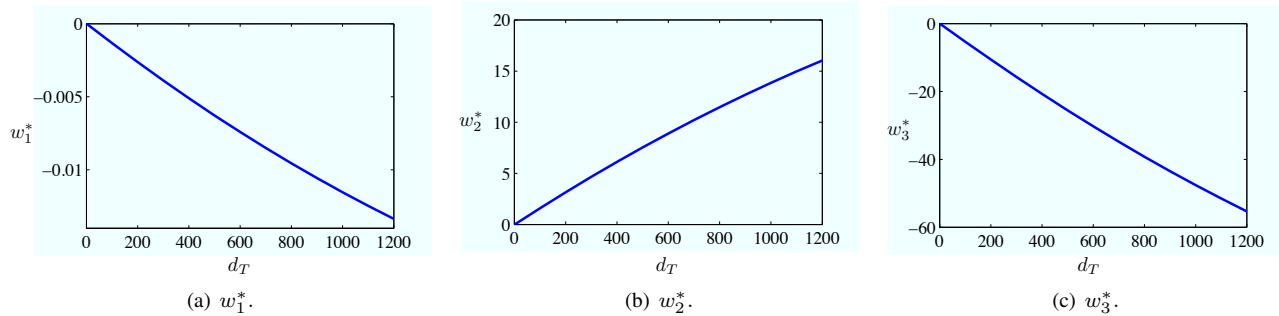

(a) w_1^*. (b) w_2^*. (c) w_3^*.

Fig. 4. Attenuation and sign-reversal in the three-stage PD cascade. Simulation is performed on the full nonlinear ODE model given by equation (2). The parameters of each stage are taken from [23] and are provided in Table 1.

bation downstream is not constant and changes fast enough compared to the dynamics of the system. Another future direction is to explore retroactivity in PD cascades with double phosphorylation, where each stage is composed of two phosphorylation reactions and two dephosphorylation reactions. Such cascades are important because they often arise in natural systems such as the MAPK cascade [4]. Finally, an important future work is experimental validation of the theory developed, especially for the case of total phosphorylated protein for which attenuation is independent of parameter values or cascade size.

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