Long Signaling Cascades Tend to Attenuate Retroactivity

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ABSTRACT Signaling pathways consisting of phosphorylation/dephosphorylation cycles with no explicit feedback allow signals to propagate not only from upstream to downstream but also from downstream to upstream due to retroactivity at the interconnection between phosphorylation/dephosphorylation cycles. However, the extent to which a downstream perturbation can propagate upstream in a signaling cascade and the parameters that affect this propagation are presently unknown. Here, we determine the downstream-to-upstream steady-state gain at each stage of the signaling cascade as a function of the cascade parameters. This gain can be made smaller than 1 (attenuation) by sufficiently fast kinase rates compared to the phosphatase rates and/or by sufficiently large Michaelis-Menten constants and sufficiently low amounts of total stage protein. Numerical studies performed on sets of biologically relevant parameters indicated that ~50% of these parameters could give rise to amplification of the downstream perturbation at some stage in a three-stage cascade. In an *n*-stage cascade, the percentage of parameters that lead to an overall attenuation from the last stage to the first stage monotonically increases with the cascade length *n* and reaches 100% for cascades of length at least 6.

INTRODUCTION

Signaling pathways are ubiquitous in living systems and cover a central role in a cell's ability to sense and respond to both external and internal input stimuli (1,2). Numerous signaling pathways consist of cycles of reversible protein modification, such as phosphorylation/dephosphorylation (PD) cycles, wherein a protein is converted, reversibly, between two forms (3). Multiple PD cycles often appear connected in a cascade fashion, such as in the MAPK cascades (4,5), and the length of the cascade has been shown to have important effects, for example, on signal amplification, signal duration, and signaling time (6–8). In particular, a wealth of work has been employing metabolic control analysis (MCA) approaches to determine analytically the amplification gains across the cascade as a small perturbation applied at the top of the cascade propagates toward the bottom stages (8–10). To our knowledge, no study has been performed on how perturbations at the bottom of a cascade propagate toward the top of the cascade.

Because cascades often intersect each other by sharing common components, such as protein substrates or kinases (11,12), perturbations at bottom or intermediate stages in a cascade can often occur. These intersections are already known to cause unwanted crosstalk between the signaling stages downstream of the intersection point (13–16). However, no attention was given to crosstalk between the stages upstream of the intersection point. Several of these works, in fact, viewed a signaling cascade as the modular composition of PD cycles, resulting in a system where the signal travels only from upstream to downstream. Theoretical work, however, has shown that PD cycles (as several other biomolecular systems) cannot be modularly connected with each other because of retroactivity effects at interconnections (17–22). Initial experimental validation of these effects on the steady-state response of a PD cycle have also appeared (23–25). These effects change the behavior of an upstream system when it is connected to its downstream clients and are relevant especially in signaling cascades, in which each PD cycle has several downstream targets. As a result of retroactivity, signaling cascades allow signals to also travel from downstream to upstream, that is, they allow bidirectional signal propagation (22,26). As a consequence, a perturbation at the bottom of the cascade can propagate to the upstream stages and have repercussions on the overall signaling.

A perturbation at the bottom of a cascade can be due to a number of factors. For example, when a downstream target or a substrate is shared with other signaling pathways, its free concentration is perturbed by these other pathways. Hence, the amount of target/substrate available to the cascade under study can suddenly change. Similarly, the introduction of an inhibitor of an active enzyme, as performed in targeted drug design, creates a perturbation at the targeted stage of the cascade.

How large is the effect of such perturbations on the upstream stages? How does the length of a cascade impact backward signal transfer?

Answering these questions will reveal the extent to which aberrant signaling in the upstream stages of a cascade can be caused by retroactivity from sharing downstream targets/substrates. It will also provide tools for targeted drug design

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by quantifying the off-target effects of inhibitors on the upstream stages.

In this article, we address these questions in cascades with a single phosphorylation cycle per stage by explicitly incorporating retroactivity in the PD cycle model. Specifically, we consider small perturbations at the bottom of the cascade and explicitly quantify, to our knowledge, for the first time how such perturbations propagate from downstream to upstream. 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 and provide sufficient conditions for attenuation. Through extensive numerical simulation, we discovered that, surprisingly, natural cascades can amplify a perturbation as it propagates upstream, but the probability of attenuation is substantially higher than that of amplification. In addition, the probability of attenuation increases with the number of stages in the cascade.

METHODS

We consider a signaling cascade composed of n phosphorylation/dephosphorylation (PD) cycles as depicted in Fig. 1. The sensitivity of response to perturbations occurring at the top of the cascade, for example in W^*_0, has been extensively studied employing MCA approaches (8–10). By contrast, here we investigate the sensitivity of response of each cycle to a perturbation at the bottom of the cascade. This perturbation can be due, for example, to an inhibitor of the active enzyme W^*_n, as it is employed in targeted drug design (27) or to the signaling from another pathway sharing a substrate with W^*_n. Our method is based on assuming a small perturbation, on linearizing the system dynamics about the steady state, and on determining the corresponding change of each cycle phosphorylated protein. Because our approach is based on linearization, it is similar in spirit to MCA approaches, which also assume small perturbations and linearize the system dynamics. Here, we are interested in determining how effectively the perturbation propagates upstream. We thus explicitly compute the sensitivity gain from one stage to the next upstream as a function of the cascade parameters.

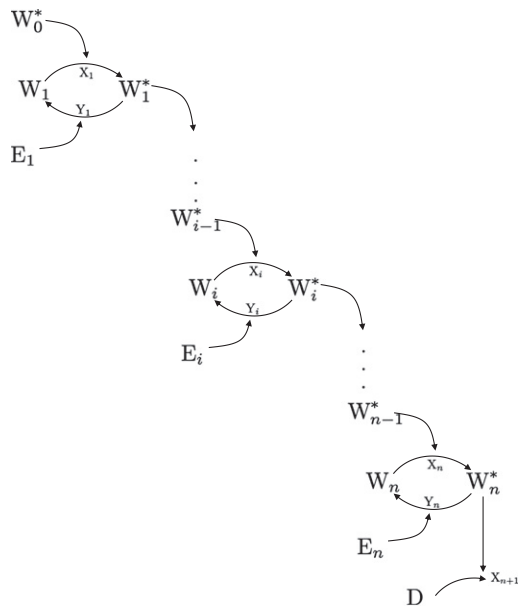

FIGURE 1 A signaling cascade with n stages of PD cycles. The phosphorylated protein W^*_{i-1} of stage i–1 functions as a kinase for protein W_i of the next stage downstream. Dephosphorylation is brought about by the phosphatase E_i. A downstream perturbation in the concentration of D, in which D can be a substrate shared with other signaling pathways or an inhibitor of the active enzyme W^*_n, results in a perturbation of protein concentration in all upstream stages.

Cascade model

At each stage i, for $i \in \{1,\ldots,n\}$, we denote by W^*_{i-1} the kinase, by E_i the phosphatase, by W_i the protein substrate, and by W^*_i 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 then turns into W^*_i. The phosphorylated protein W^*_i is, in turn, a kinase for the next cycle and binds to downstream substrates, forming the complex X_{i+1}. The phosphatase E_i activates the dephosphorylation of the protein W^*_i by binding to W^*_i and forming the complex Y_i. This complex is in turn converted to W_i. We employ the following two-step reaction model for each phosphorylation and dephosphorylation reaction (28,29) at stage $i \in \{1,\ldots,n\}$ of the cascade:

$$W_i + W^*_{i-1} \underset{\bar{a}_i}{\overset{a_i}{\rightleftharpoons}} X_i \xrightarrow{k_i} W^*_i + W^*_{i-1},$$

$$W^*_i + E_i \underset{\bar{b}_i}{\overset{b_i}{\rightleftharpoons}} Y_i \xrightarrow{\bar{k}_i} W_i + E_i.$$

We assume that protein W_i and phosphatase E_i are conserved at every stage, and are in total amounts W_{iT} and E_{iT}, respectively. Therefore, we have the conservation relations

$$\begin{aligned} W_i + W^*_i + X_i + Y_i + X_{i+1} &= W_{iT}, \\ E_i + Y_i &= E_{iT}, \end{aligned} \tag{1}$$

in which, for a species X, we have denoted by X its concentration. We assume that the input kinase to the first stage, W^*_0, is produced at rate $k(t)$ and decays at rate δ, that is,

$$W^*_0 \underset{k(t)}{\overset{\delta}{\rightleftharpoons}} \varnothing.$$

Finally, we assume that the output protein of the last stage, W^*_n, reacts with species D downstream of the cascade. These species D can model, for example, a signaling molecule or an inhibitor of the active enzyme W^*_n (a drug), such as considered in targeted drug design (27), in which the total concentration of D can be perturbed, for example, by adding more drug. Species D can also model a substrate that is shared with other signaling pathways. In this case, D is a substrate for another active enzyme, say S, whose concentration is controlled by another signaling cascade. Hence, the amount of free D plus the amount of D bound to W^*_n, which we call D_T, can be perturbed (it can increase or decrease) by a change in the concentration of the active enzyme S. Denoting by X_{n+1} the complex formed by W^*_n and D, we have that

$$W^*_n + D \underset{\bar{a}_{n+1}}{\overset{a_{n+1}}{\rightleftharpoons}} X_{n+1} \text{ with } D_T \triangleq D + X_{n+1}.$$

In this study, we consider D_T as the parameter to be perturbed and calculate the sensitivity of the steady-state response of each cycle's active protein to small perturbations in D_T.

The differential equations that describe the dynamics of the cascade are given, for $i \in \{1,\ldots,n\}$, by

$$\begin{aligned}
\dot{W}_0^* &= -\delta W_0^* + k(t) - \boxed{\left(a_1 W_0^* W_1 - (\overline{a}_1 + k_1)X_1\right)} \\
\dot{X}_i &= a_i W_{i-1}^* W_i - (\overline{a}_i + k_i)X_i \\
\dot{W}_i^* &= k_i X_i - b_i W_i^* E_i + \overline{b}_i Y_i \\
&\quad - \boxed{\left(a_{i+1} W_i^* W_{i+1} - (\overline{a}_{i+1} + k_{i+1})X_{i+1}\right)} \\
\dot{Y}_i &= b_i W_i^* E_i - (\overline{b}_i + \overline{k}_i)Y_i \\
\dot{W}_n^* &= k_n X_n - b_n W_n^* E_n + \overline{b}_n Y_n - \boxed{\left(a_{n+1} D W_n^* - \overline{a}_{n+1} X_{n+1}\right)} \\
\dot{X}_{n+1} &= a_{n+1} D W_n^* - \overline{a}_{n+1} X_{n+1}.
\end{aligned}$$

Recognizing 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 (Eq. 1), we obtain for $i \in \{1,\ldots,n\}$ that

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

Perturbation analysis

In this section, we consider the cascade to be at the steady state and investigate how a small perturbation in the concentration D_T perturbs the steady-state concentrations at every stage of the cascade. We denote the steady-state value of the upstream input $k(t)$ by $\overline{k}$ and that of D_T by $\overline{D}_T$. The corresponding equilibrium values of the protein concentrations

$$W_0^*, W_i^*, X_i, Y_i, W_i,$$

for $i \in \{1,\ldots,n\}$, and X_{n+1} are denoted by

$$\overline{W}_0^*, \overline{W}_i^*, \overline{X}_i, \overline{Y}_i, \overline{W}_i,$$

for $i \in \{1,\ldots,n\}$, and $\overline{X}_{n+1}$, respectively. We represent the perturbation of D_T with respect to its steady-state value by $d_T = D_T - \overline{D}_T$. Note that if $d_T > 0$, the downstream perturbation is positive, that is, the concentration D_T increases. If instead $d_T < 0$, the downstream perturbation is negative, that is, the concentration D_T decreases. Hence, both positive and negative perturbations are considered. The corresponding perturbations of the states of the cascade about the equilibrium values

$$\overline{W}_0^*, \overline{W}_i^*, \overline{X}_i, \overline{Y}_i,$$

for $i \in \{1,\ldots,n\}$, and $\overline{X}_{n+1}$ are denoted by

$$w_0^*, w_i^*, x_i, y_i,$$

for $i \in \{1,\ldots,n\}$, and x_{n+1}, respectively. Similarly, denote by Z_i for $i \in \{1,\ldots,n\}$ the concentration of the total phosphorylated protein at stage i, that is, $Z_i = W^*_i + Y_i + X_{i+1}$. Denote the corresponding perturbation about the steady state

$$\overline{Z}_i = \overline{W}_i^* + \overline{Y}_i + \overline{X}_{i+1}$$

by z_i, which can be written as

$$z_i = w_i^* + y_i + x_{i+1}$$

for all $i \in \{1,\ldots,n\}$.

The linearization of the system in Eq. 2 about the equilibrium

$$\overline{W}_0^*, \overline{W}_i^*, \overline{X}_i, \overline{Y}_i, \text{and } \overline{X}_{n+1}$$

for $i \in \{1,\ldots,n\}$ is given by

$$\begin{aligned}
\dot{w}_0^* &= -\delta w_0^* - \dot{x}_1 \\
\dot{x}_i &= a_i \overline{W}_i w_{i-1}^* + a_i \overline{W}_{i-1}^* \left(-w_i^* - x_i - y_i - x_{i+1}\right) \\
&\quad - (\overline{a}_i + k_i)x_i \\
\dot{w}_i^* &= k_i x_i + b_i \overline{W}_i^* y_i - b_i \overline{E}_i w_i^* + \overline{b}_i y_i - \dot{x}_{i+1} \\
\dot{y}_i &= -b_i \overline{W}_i^* y_i + b_i \overline{E}_i w_i^* - (\overline{b}_i + \overline{k}_i)y_i \\
\dot{x}_{n+1} &= a_{n+1} \overline{D} w_n^* - a_{n+1} \overline{W}_n^* x_{n+1} - \overline{a_{n+1}} x_{n+1} + a_{n+1} \overline{W}_n^* d_T,
\end{aligned} \tag{3}$$

in which we have for $i \in \{1,\ldots,n\}$ that (from setting the time derivatives in the expressions in Eq. 2 equal to zero)

$$\overline{W}_0^* = \frac{\overline{k}}{\delta}, \tag{4}$$

$$\overline{W}_i = K_i \frac{\overline{k}_i}{k_i} \frac{\overline{E}_i}{\overline{W}_i^* + \overline{K}_i} \left(1 + \frac{\overline{W}_i^*}{\overline{K}_i}\right) \frac{\overline{W}_i^*}{\overline{W}_{i-1}^*}, \tag{5}$$

$$\overline{Y}_i = \frac{E_{iT}}{1 + \overline{K}_i / \overline{W}_i^*}, \tag{6}$$

$$\overline{X}_i = \frac{\overline{k}_i}{k_i} \overline{Y}_i, \tag{7}$$

in which

$$\overline{K}_i = \frac{\overline{b}_i + \overline{k}_i}{b_i}$$

is the Michaelis-Menten constant of the dephosphorylation reaction, while

$$K_i = \frac{\overline{a}_i + k_i}{a_i}$$

is the Michaelis-Menten constant of the phosphorylation reaction.

Because we are interested in the steady-state values of w^*_i, we set the time derivatives to zero in system in Eq. 3 to obtain

$$x_i = \frac{\overline{k}_i}{k_i} \tilde{E}_i w_i^*, \tag{8}$$

$$w_i^* = T_i \left(\overline{W}_i w_{i-1}^* - \overline{W}_{i-1}^* x_{i+1}\right), \tag{9}$$

for $i \in \{1,\ldots,n\}$, in which

$$\tilde{E}_i \triangleq \frac{\overline{K}_i E_{iT}}{\left(\overline{W}_i^* + \overline{K}_i\right)^2}, \tag{10}$$

$$T_i \triangleq \frac{1}{\overline{W}_{i-1}^* + \tilde{E}_i \left(\overline{W}_{i-1}^* + \frac{\overline{k}_i}{k_i}\left(\overline{W}_{i-1}^* + K_i\right)\right)}. \tag{11}$$

Fig. 2 represents Eqs. 8 and 9 in a block diagram form, which highlights the directionality of signal propagation through the stages in the cascade. Basically, the perturbation d_T propagates upstream in the cascade through

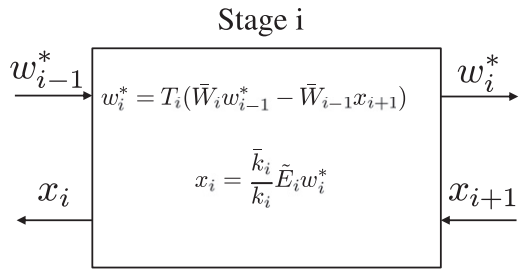

FIGURE 2 A block diagram representation of the steady-state response of stage i to a small downstream perturbation in D_T. The downstream perturbation propagates upstream through perturbations x_i in the complexes of active proteins with their downstream substrates.

perturbations in the concentrations X_i. Hence, in this steady-state response model, retroactivity is due to the complex X_i of the active protein with its downstream substrate.

RESULTS

Analytical results

Referring to Fig. 2, the perturbation d_T propagates upstream through perturbations x_i and causes perturbations z_i and w^*_i in the total and free phosphorylated protein concentrations, respectively, at every stage. How do these perturbations transfer from one stage of the cascade to the next one upstream?

To answer this question, we calculate the gains

$$\Phi_i = \frac{|z_i|}{|z_{i+1}|} \text{ and } \Psi_i = \frac{|w^*_i|}{|w^*_{i+1}|}$$

at every stage i. A gain >1 means that small perturbations are amplified as they transfer from downstream to upstream, while a gain <1 means that small perturbations are attenuated as they transfer from downstream to upstream.

Because $|z_i| = \Phi_i |z_{i+1}|$, we have that

$$|z_1| = \left(\prod_{i=1}^{n-1} \Phi_i\right)|z_n|,$$

where $\prod$ denotes multiplication. We thus define the total gain Φ_{tot} from stage n to stage 1 as

$$\Phi_{tot} = \prod_{i=1}^{n-1} \Phi_i.$$

Similarly, the total gain Ψ_{tot} from stage n to stage 1 is defined as

$$\Psi_{tot} = \prod_{i=1}^{n-1} \Psi_i.$$

Having a total gain <1 means that, overall, the cascade attenuates downstream perturbations, even if some stages may amplify the perturbation.

We first focus on the gains Φ_i of total active protein concentration. The total active protein concentration can be experimentally determined by measuring protein activity through phosphospecific antibodies (30). By contrast, the free active protein may be more difficult to measure. When it is an active transcription factor, it can be measured indirectly, for example, by placing a reporter gene under the control of the promoter that it regulates. The expression of the gain Φ_i at each stage i can be explicitly calculated as a function of the cascade parameters from the relations in the block diagram of Fig. 2 (see the Supporting Material). This expression is given by

$$\Phi_i = \left(\frac{\tilde{E}_i\frac{\bar{k}_i}{k_i} + F_i}{1 + \tilde{E}_i + \tilde{E}_i\frac{\bar{k}_i}{k_i} + F_i}\right) \times \left(\frac{\frac{\bar{k}_{i+1}}{k_{i+1}}\tilde{E}_{i+1}}{\frac{\bar{k}_{i+1}}{k_{i+1}}\tilde{E}_{i+1} + F_{i+1}}\right) \text{for all } i \in \{1, \ldots, n-1\},$$

in which F_i and F_{i+1} are positive quantities. Because

$$\frac{\tilde{E}_i\frac{\bar{k}_i}{k_i} + F_i}{1 + \tilde{E}_i + \tilde{E}_i\frac{\bar{k}_i}{k_i} + F_i} < 1$$

and

$$\frac{\frac{\bar{k}_{i+1}}{k_{i+1}}\tilde{E}_{i+1}}{\tilde{E}_{i+1}\frac{\bar{k}_{i+1}}{k_{i+1}} + F_{i+1}} < 1,$$

we have that

$$\Phi_i < 1, \quad \text{for all } i \in \{1, \ldots, n-1\}.$$

Furthermore, we have that (see the Supporting Material)

$$sign(z_i) = -sign(z_{i+1}) \text{for all } i \in \{1, \ldots, n-1\},$$

that is, an increase of Z_{i+1} implies a decrease of Z_i. Therefore, there is a sign reversal of the perturbation on the total phosphorylated protein concentration across the stages and the magnitude of the perturbation at every stage is always attenuated as it propagates upstream in the cascade. That is, $|z_1| < |z_2| < \ldots < |z_{n-1}| < |z_n|$ for all parameter values. Furthermore, this implies also that we have overall attenuation from downstream to upstream in the cascade, that is, $\Phi_{tot} < 1$. Because these facts do not depend on the specific parameter values or the length of the cascade, they highlight a new structural property of signaling cascades.

For the perturbation on the free active protein concentration, we also have that (see the Supporting Material)

$$sign\left(w_i^*\right) = -sign\left(w_{i+1}^*\right) \text{for all } i \in \{1, \ldots, n-1\},$$

that is, when the perturbation w^*_{i+1} is positive the next upstream stage has a perturbation w^*_i with negative sign. Hence, if the downstream perturbation causes a decrease of the active protein concentration at one stage, it causes an increase of the active protein concentration in the next upstream stage. An expression of the stage gain Ψ_i can be calculated as a function of the cascade parameters starting from the relations of the block diagram of Fig. 2. The exact expression is calculated in the Supporting Material and it is such that

$$\Psi_i \leq \frac{\dfrac{\overline{k}_{i+1}}{k_{i+1}} \dfrac{E_{(i+1)T}}{\overline{K}_{i+1}}}{1 + \dfrac{E_{iT}}{(\overline{K}_i + W_{iT})\left(1 + \dfrac{W_{iT}}{\overline{K}_i}\right)}\left(1 + \dfrac{\overline{k}_i}{k_i}\left(1 + \dfrac{K_i}{W_{(i-1)T}}\right)\right)}. \tag{12}$$

Therefore, one can control the amount of attenuation/amplification through the cascade parameters as follows. The smaller the $W_{(i-1)T}$, the more the attenuation from stage $i+1$ to i (i.e., the smaller the upper bound on Ψ_i in Eq. 12). Moreover, sufficiently large values of K_i and $\overline{K}_i$ for all i lead to an increased attenuation at every stage. In turn, large K_i and $\overline{K}_i$ and small W_{iT} are responsible for a decreased sensitivity of the response of stage i to upstream stimuli (29). As a consequence, a more graded upstream-to-downstream response at all stages is associated with an increased attenuation of downstream perturbations.

From the expressions in Eq. 12, it also follows that a sufficient condition for having attenuation at stage i of the downstream perturbation is that

$$\frac{\overline{k}_{i+1}}{k_{i+1}} \frac{E_{(i+1)T}}{\overline{K}_{i+1}} < 1.$$

This condition is valid for general PD cascades. However, it has a particularly simple meaning in the case in which the signaling pathway is weakly activated as explained in what follows. In Heinrich et al. (6), it was found that a requirement for upstream-to-downstream signal amplification is that the phosphorylation rate constant should be larger than the dephosphorylation rate constant. For a weakly activated pathway with $K_i \gg W_{(i-1)T}$, the phosphorylation rate constant is well approximated by $\alpha_i = k_i W_{iT}/K_i$ (see the Supporting Material). In the case in which $\overline{K}_i \gg W_{iT}$, the dephosphorylation rate constant is well approximated by $\beta_i = \overline{k}_i E_{iT}/\overline{K}_i$ (see the Supporting Material). As a consequence, to have upstream-to-downstream signal amplification, it is required that $\alpha_i > \beta_i$, which, when $K_i \geq W_{iT}$, implies that

$$\frac{\overline{k}_i}{k_i} \frac{E_{iT}}{\overline{K}_i} < 1.$$

This, in turn, implies that $\Psi_{i-1} < 1$ and hence that the downstream perturbation is attenuated as it transfers from stage i to stage $i-1$. Hence, in weakly activated pathways in which $K_i \geq W_{iT}$, $\overline{K}_i \gg W_{iT}$, and $K_i \gg W_{(i-1)T}$, upstream-to-downstream signal amplification is associated with attenuation of downstream perturbations as they transfer upstream. This, in turn, implies unidirectional signal propagation from upstream to downstream.

From the expressions in Eq. 12, it also follows that a necessary condition for having $\Psi_i > 1$, that is, for amplifying a downstream perturbation as it transfers from stage $i+1$ to stage i, is that

$$\frac{\overline{k}_{i+1}}{k_{i+1}} \frac{E_{(i+1)T}}{\overline{K}_{i+1}} > 1.$$

This condition, in turn, in the case in which $\overline{K}_{i+1} \gg W_{(i+1)T}$, $W_{(i+1)T} \leq K_{i+1}$, and $K_{i+1} \gg W_{iT}$ implies that the phosphorylation rate constant α_{i+1} is smaller than the dephosphorylation rate constant β_{i+1}. As a consequence, there is no amplification at stage $i+1$ of the signal traveling from upstream to downstream as the required condition for amplification as determined by Heinrich et al. (6) is violated. Hence, in weakly activated pathways in which $K_{i+1} \geq W_{(i+1)T}$, $\overline{K}_{i+1} \gg W_{(i+1)T}$, and $K_{i+1} \gg W_{iT}$ if a downstream perturbation is amplified as it propagates from stage $i+1$ to stage i, then there is no amplification from stage i to stage $i+1$ for the signal traveling from upstream to downstream in response to a stimulus at the top of the cascade.

From the expressions of Ψ_i, we can also derive a necessary condition for attenuation (see the Supporting Material). Specifically, to have $\Psi_i < 1$ at stage i, it is necessary that

$$\frac{\dfrac{\overline{k}_{i+1}}{k_{i+1}} \dfrac{\overline{K}_{i+1} E_{(i+1)T}}{\left(\overline{W}^*_{i+1} + \overline{K}_{i+1}\right)^2}}{1 + \dfrac{\overline{K}_i E_{iT}}{\left(\overline{W}^*_i + \overline{K}_i\right)^2}\left[1 + \dfrac{\overline{k}_i}{k_i}\left(1 + \dfrac{K_i}{\overline{W}^*_{i-1}}\left(1 + \left(1 + \dfrac{\overline{W}^*_i}{\overline{K}_i}\right)\dfrac{\overline{W}^*_i}{\overline{W}^*_{i-1}}\right)\right)\right]} < 1. \tag{13}$$

If the necessary condition is violated at stage i, then either stage $i-1$ or stage i amplify the downstream perturbation. This expression can be employed to determine parameter values for which amplification of the downstream perturbation can result at any given stage and can be useful to determine the efficacy of the off-target effects of an inhibitor.

To conclude the analytical study, we investigate how d_T affects w^*_n and z_n. It can be shown (see the Supporting Material) that $|w^*_n| < |d_T|$ and that $|z_n| < |d_T|$. That is, the perturbation d_T induces changes w^*_n and z_n about $\overline{W}^*_n$ and $\overline{Z}_n$, respectively, that are less than d_T in magnitude, regardless of the parameters. Also, we have that $sign(d_T) = -sign(w^*_n)$ and $sign(d_T) = sign(z_n)$.

Numerical results

In this section, we first illustrate the results on a three-stage cascade example. We then employ the analytically computed expressions Ψ_i to determine the probability that natural cascades attenuate a downstream perturbation as it transfers upstream in the cascade. We finally study the effect of the length of the cascade on the overall gain $\Psi_{tot.}$ All simulations are performed on the full nonlinear model of the system in Eq. 2 in MATLAB (The MathWorks, Natick, MA) using the built-in ODE23s solver.

Fig. 3 shows how the perturbation propagates upstream in a three-stage cascade for the parameter values of Huang and Ferrell (28). This figure illustrates that, surprisingly, the relationship between w^*_i and d_T is approximately linear even for large perturbations d_T (up to 400 nM). Hence, the theoretical results must hold. In particular, the values of w^*_1 and w^*_3 are negative whereas the value of w^*_2 is positive. That is, the perturbation on W^*_i switches sign from one stage to the next upstream. The gains Ψ_i calculated from the expression in the Supporting Material for the parameter values of Huang and Ferrell (28) are given by $\Psi_1 = 2.45 \times 10^{-5}$ and $\Psi_2 = 2.14 \times 10^{-2}$. Because Ψ_1 and Ψ_2 are both <1, the cascade should attenuate the downstream perturbation at every stage. This is confirmed by Fig. 3 in which for the same value of d_T, we have that $|w^*_i|$ becomes smaller and smaller as the stage i decreases (i.e., as the perturbation propagates upstream). Because the values of Ψ_i are $\ll 1$, this three-stage cascade practically enforces unidirectional signal propagation from upstream to downstream. Note that as long as the applied perturbation d_T is small enough, the relationship between d_T and w^*_i is linear and hence all our results hold independently of the parameter values. Additional examples for different parameter values are provided in the Supporting Material.

To validate the necessary condition for attenuation at stage i, we constructed a parameter set that violates the necessary condition for attenuation (see Eq. 13). In this case, we should expect that at the stage i for which $\Psi_i > 1$, the downstream perturbation is amplified, that is, $|w^*_i| > |w^*_{i+1}|$. The necessary condition in Eq. 13 can be violated by choosing phosphatase amounts that increase with the stage number, that is, $E_{1T} \ll E_{2T} \ll E_{3T}$ and substrate amounts that decrease with the stage number, that is, $W_{1T} \gg W_{2T} \gg W_{3T}$. We utilized these conditions and constructed a cascade that amplifies downstream perturbations. The result is shown in Fig. 4. The resulting parameter values are still biologically meaningful as they are contained in the parameter intervals estimated in Huang and Ferrell (28). Therefore, these cascades are capable of also transmitting a perturbation from downstream to upstream by amplifying its amplitude.

Do natural signaling cascades attenuate downstream perturbations?

To determine the probability that a natural signaling cascade attenuates or amplifies downstream perturbations, we evaluated the expression of the gains Ψ_i on parameters extracted with uniform probability distribution from intervals taken from the literature (28,31–33). We present the results first for a three-stage cascade starting from conservative intervals and we progressively reduce the size of the intervals. In all cases, each parameter has a range and a uniform probability distribution is used to sample parameters for each range. Also, even though the range of parameters for each cycle is the same, in the simulations each cycle has different parameters (randomly picked from the given range).

Conservative intervals

In this case, we randomly chose parameters through a uniform probability distribution from the intervals given in Table 1. The maximum and minimum values of the intervals were chosen to be the maximum and minimum of the union of the intervals defined in Huang and Ferrel (28) and Bhalla and Iyengar (31). This is a conservative way of choosing the intervals as the parameters of Huang and Ferrell (28) and Bhalla and Iyengar (31) are taken from different organisms. In selecting the range for D_T, we assumed that D is a downstream protein substrate and thus its interval of variation was chosen to be the same as that for $W_{iT.}$

We simulated the three-stage cascade 10,000 times and the results are reported in Table 2. This table shows the percentage of simulations that resulted in $\Psi_i > 1$ for every

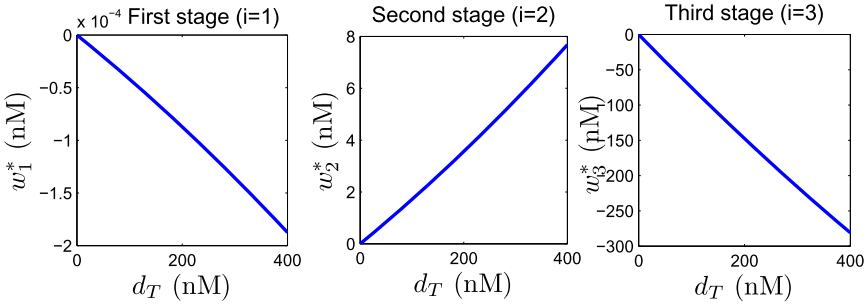

FIGURE 3 Attenuation and sign-reversal in a three-stage cascade. The x axis shows the value of the perturbation d_T and the y axis shows the steady-state value of the resulting perturbations w^*_1, w^*_2, and w^*_3. Simulation is performed on the full nonlinear ODE model given by Eq. 2. The parameters of each stage i are taken from Huang and Ferrell (28) and are given by $k_i = 150\ (\text{min})^{-1}$, $\overline{k}_i = 150\ (\text{min})^{-1}$, $\alpha_i = 2.5\ (\text{nM min})^{-1}$, $\overline{a}_i = 600\ (\text{min})^{-1}$, $b_i = 2.5\ (\text{nM min})^{-1}$, $\overline{b}_i = 600(\text{min})^{-1}$, $E_{3T} = 120$ 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, and $\overline{D}_T = 0$ nM. As a result, $K_i = 300$ nM and $\overline{K}_i = 300$ nM.

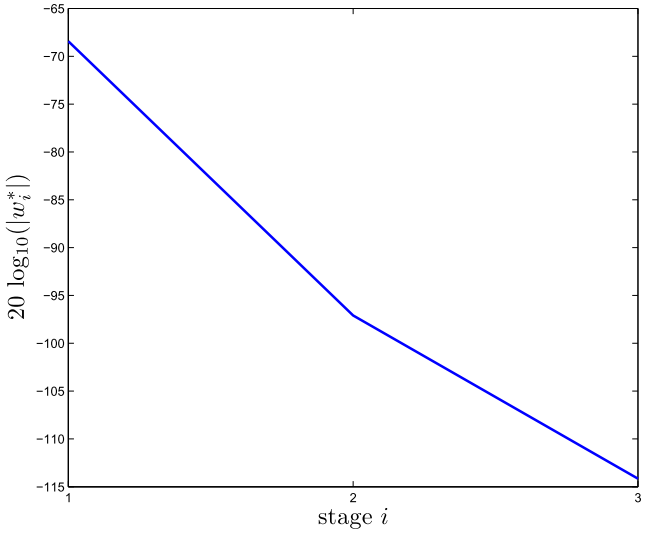

FIGURE 4 Amplification in a three-stage cascade. Numerical simulation of system in Eq. 2: value of $|w^*_i|$ for $i \in \{1,\ldots,n\}$ in response to a unit perturbation $d_T = 1$. This plot shows that violation of the necessary condition leads to amplification of the downstream perturbation as it transfers upstream in the cascade. Parameters of stage i are given by: $k_i = 150$ $(\text{min})^{-1}$, $\overline{k}_i = 150$ $(\text{min})^{-1}$, $a_i = 2500$ $(\text{nM min})^{-1}$, $\overline{a}_i = 600$ $(\text{min})^{-1}$, $b_i = 2500$ $(\text{nM min})^{-1}$, $\overline{b}_i = 600$ $(\text{min})^{-1}$, $E_{3T} = 120$ nM, $E_{2T} = 30$ nM, $E_{1T} = 0.3$ nM, $W_{3T} = 3$ nM, $W_{2T} = 30$ nM, $W_{1T} = 1200$ nM, $\overline{W}^*_0 = 0.3$ nM, and $\overline{D}_T = 0.9$ nM.

$i \in \{1,2\}$, that is, that resulted in attenuation at stage i. The probability of stage 1 attenuating the downstream perturbation is 71.34% and the probability of stage 2 attenuating it is 55%. Moreover, because the probability that $\Psi_{tot} < 1$ is 79.4%, the probability of such cascades providing an overall attenuation of a downstream perturbation is quite high. To explore whether 10,000 simulations were enough to obtain meaningful probability figures, we calculated at each new simulation the percentage of all performed simulations that resulted in attenuation. The probabilities converge for every stage to the values given in Table 2; hence, performing more simulations will not significantly change the results (see the Supporting Material).

TABLE 1 Conservative intervals

Parameter	Interval for simulation	Interval from Huang and Ferrell (28)	Interval from Bhalla and Iyengar (31)
k_i, $\overline{k}_i$	[6.3, 600]	[150, 150]	[6.3, 600]
a_i, b_i	[18.018, 4545.45]	[2500, 2500]	[18.018, 4545.45]
$\overline{a}_i$, $\overline{b}_i$	[25.2, 2400]	[600, 600]	[25.2, 2400]
E_{iT}	[0.3, 224]	[0.3, 120]	[3.2, 224]
W_{iT}	[3, 1200]	[3, 1200]	[180, 360]
$\overline{W}^*_0$	[0.3, 100]	[0.3, 0.3]	[100, 100]
$\overline{D}_T$	[0, 1200]	—	—

For each of the parameters of the cascade, we indicate the interval considered for simulation and the intervals given in Huang and Ferrel (28) and Bhalla and Iyengar (31). For simulation, a uniform probability distribution over each interval is chosen to sample parameter values. Also, each stage has different parameters even though all were extracted from a uniform probability distribution.

TABLE 2 Three-stage cascade attenuation percentage

	Ψ_1	Ψ_2	Ψ_{tot}
% of $\Psi_i < 1$	71.34	55	79.4

The parameters are taken randomly from Table 1.

Intervals based on Bhalla and Iyengar (31)

We considered the nominal parameter values given in Bhalla and Iyengar (31) and then constructed intervals by varying these values by 20, 50, and 80%. Specifically, for every parameter with nominal value p, we considered a confidence interval of the form $[(1 - 0.x)\ p,\ (1 + 0.x)\ p]$ for the three different cases in which $x = 2$, $x = 5$, and $x = 8$. The results for these three different cases are shown in Table 3. Even when the parameters are allowed to vary by 80% from the nominal values, the probability that any given stage attenuates the perturbation is very high and the probability that the cascade provides overall attenuation (i.e., $\Psi_{tot} < 1$) is 1. As performed in the previous case, the results of Table 3 are obtained performing 10,000 numerical simulations. In the Supporting Material, we show that this number is large enough to attain convergence of the probabilities.

Intervals based on Levchenko et al. (32)

We next considered the nominal parameter values given in Levchenko et al. (32) and constructed intervals by varying these values by 20, 50, and 80%. Specifically, for every parameter with nominal value p, we considered a confidence interval of the form $[(1 - 0.x)\ p,\ (1 + 0.x)\ p]$ for the three different cases in which $x = 2$, $x = 5$, and $x = 8$. The results for these three different cases are shown in Table 4. When the parameters are allowed to change by 50% with respect to the nominal values, the probability of attenuation at each stage is lower than the values obtained for the parameters of Bhalla and Iyengar (31) (Table 3). With 80% parameter variation, there is a significant percentage of the possible parameters (10%) that allows us to amplify, overall, the downstream perturbation from stage 3 to stage 1. Moreover, 50% of the parameters led to having $\Psi_1 > 1$ or $\Psi_2 > 1$ and only 2.2% of the parameters led to having both $\Psi_1 > 1$ and $\Psi_2 > 1$. Therefore, 50% of the possible parameter

TABLE 3 Three-stage cascade attenuation percentage for different intervals near the nominal parameter values of Bhalla and Iyengar (31)

	Ψ_1	Ψ_2	Ψ_{tot}
% of $\Psi_i < 1$ with 20% variation	100	100	100
% of $\Psi_i < 1$ with 50% variation	99.98	100	100
% of $\Psi_i < 1$ with 80% variation	96.895	99.91	100

TABLE 4 Three-stage cascade attenuation percentage for different intervals near the nominal parameter values of Levchenko et al. (32)

	Ψ_1	Ψ_2	Ψ_{tot}
% of $\Psi_i < 1$ with 20% variation	77.49	100	100
% of $\Psi_i < 1$ with 50% variation	65.85	93.32	97.07
% of $\Psi_i < 1$ with 80% variation	64.69	82.68	90.91

values lead to amplification in at least one stage in the cascade. The results of Table 4 are obtained performing 10,000 numerical simulations. The Supporting Material shows that, by the time the 10,000th simulation is performed, the probability has converged to its final value.

We then analyzed how the length n of the cascade affects the overall attenuation from stage n to stage 1, that is, how it affects the gain $\Psi_{tot.}$ To perform this study, we first simulated a 10-stage cascade 10,000 times with the same parameter ranges as given in Table 1. The result is shown in Table 5. The probability of the last two stages ($i = 8,9$) attenuating the perturbation has significantly increased compared to the three-stage case (Table 2). Furthermore, the probability of overall attenuation, that is, that $\Psi_{tot} < 1$, is 1. Hence, even when some stages amplify the downstream perturbation, the rest of the stages provide attenuation so that the overall attenuation in the cascade is much more than the overall amplification. To confirm that 10,000 simulations were enough to provide meaningful probability figures, we analyzed the convergence of the probability after each simulation run in the Supporting Material.

Finally, to study how the number of stages in a cascade impacts the probability of overall attenuation, that is, the probability that $\Psi_{tot} < 1$, we performed a number of numerical simulations extracting parameters from the intervals of Table 1 for cascades with increasing number of stages. The probability of overall attenuation monotonically increases as the number of stages in the cascade increases and it reaches 100% for cascades of length at least 6 (Fig. 5). For each number of stages, n, we performed a sufficiently large number of simulations for different values of the parameters sampled in the intervals of Table 1 (see the Supporting Material). This result implies that for a fixed range of parameters, adding more stages contributes significantly to the probability of overall attenuation from stage n to stage 1. For example, the probability of a three-stage cascade providing overall attenuation was found to be 79.4% while, for the same range of parameters, the probability of a 10-stage cascade providing overall attenuation was found to be 100%.

DISCUSSION

Upstream-to-downstream signal transfer in signaling cascades determines how external stimuli at the top of the cascade, such as growth factors, hormones, and neurotransmitters, affect downstream targets, such as gene expression. Several works focused on determining the sensitivity of each stage of a cascade to small perturbations at the top of the cascade. In these studies, it was found that multiple stages in the cascade can boost the overall cascade sensitivity to upstream input stimuli (8–10). Downstream-to-upstream signal transfer, on the other hand, determines how a perturbation at the bottom of the cascade due, for example, to a drug or to sharing a substrate with another signaling pathway, affects the upstream stages of the cascade. This has not been studied before.

Here, we have studied for the first time (to our knowledge) the response of each stage of a cascade to small perturbations in a substrate or inhibitor at the bottom of the cascade. One of our results is that larger numbers of stages in the cascade lead to higher overall attenuation of the signal transfer from downstream to upstream. This provides another reason why natural signaling cascades are usually composed of multiple stages: more stages enforce unidirectional signal propagation, which is certainly desirable in any natural or human-made signal transmission system.

We have computed analytical expressions of the downstream-to-upstream gains at each stage of the cascade as a function of the cascade parameters. These expressions uncover two main structural properties of signaling cascades, which are independent of the specific parameter values.

First, the perturbation on the total or free active protein concentration switches sign at each stage of the cascade as it propagates upstream. That is, if at one stage the amount of free or total active protein increases because of the perturbation, it must decrease at the next upstream stage.

Second, the perturbation on the total amount of active protein is attenuated as it propagates from one stage to the next one upstream. By contrast, the way the perturbation propagates on the free amount of active protein depends on the specific parameter values. We have provided a sufficient condition for attenuation, which applies to general PD cascades and has a particularly simple meaning in the special case of weakly activated pathways. That is, for weakly activated pathways in which each cycle operates in the hyperbolic regime, amplification of a perturbation at the top of the cascade as it propagates downstream implies attenuation of a perturbation at the bottom of the cascade as it propagates upstream.

TABLE 5 Ten-stage cascade attenuation percentage for the parameter values in Table 1

i	1	2	3	4	5	6	7	8	9	Ψ_{tot}
% of $\Psi_i < 1$	67.3	71.8	72.9	73.3	73.7	74.5	72.9	76.2	59.8	100

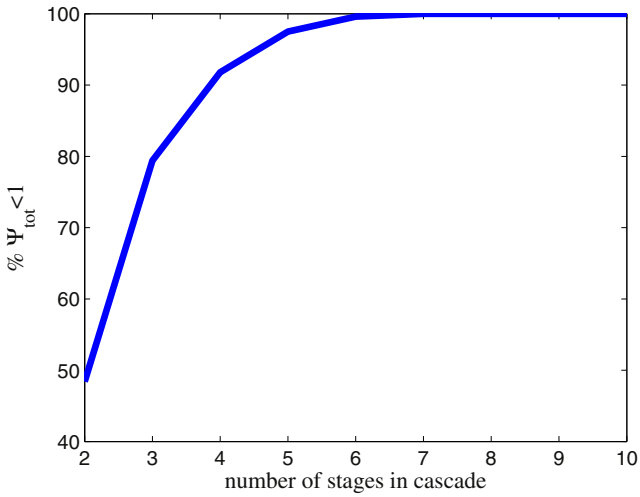

FIGURE 5 Percentage of simulations with overall attenuation ($\Psi_{tot} < 1$) as a function of the number of stages in a cascade with parameters randomly selected from the intervals of Table 1.

Although simulation studies performed in Ventura et al. (22) suggested that a perturbation is attenuated as it propagates upstream in the cascade, the analytical expressions of the gains found in this article clearly show that amplification of the perturbation on the free protein concentration is also possible. To understand whether natural signaling cascades are more likely to attenuate or to amplify a downstream perturbation on the free active protein concentration, we performed a numerical study. In this study, the gain Ψ_i at each stage was computed with parameter values randomly extracted from biologically meaningful sets obtained from the literature (28,31–33). This numerical study reveals that signaling cascades are substantially more likely to attenuate a downstream perturbation than to amplify it and that longer signaling cascades have a higher probability of overall attenuation. However, in signaling cascades of length 3, which is the most common length found in practice, ~50% of the biologically meaningful parameters taken from Levchenko et al. (32) lead to amplification at least at one stage and ~10% of them resulted in overall amplification (from stage 3 to stage 1).

In summary, our findings suggest that the effects of crosstalk between signaling pathways sharing common components can be felt even upstream of the common component as opposed to only downstream of it as previously believed. We believe this provides a new mechanism by which a pathway can become overactivated as found in several pathological conditions such as cancer (13–16). At the same time, our study provides tools to understand how the effects of a targeted drug (26,27) may propagate to obtain off-target effects and how these effects depend on the cascade parameters.

This article addresses cascades in which, at each stage, there is a single phosphorylation cycle. However, several natural cascades, such as the MAPK cascade, display double phosphorylation and experimental work performed in *Drosophila* embryos has demonstrated that a perturbation in one of the substrates at the bottom of the cascade affects the phosphorylation level at the last cycle of the cascade (24). Whether such a perturbation can propagate on the higher levels of the cascade was not addressed. In future work, we thus plan to extend our gain calculations to cascades with double phosphorylation in order to establish the extent to which such perturbations propagate on the higher levels of the MAPK cascade. It was shown in previous work that the presence of double phosphorylation can lead to sustained oscillations even in the absence of explicit negative feedback (34). In such instances, our analysis will have to extend to dynamic perturbations as opposed to static perturbations in order to understand how these oscillations propagate upstream in the cascade.

Recently published experimental articles clearly show that perturbations in the downstream targets of a signaling cascade cause a perturbation in the immediate upstream signaling stage. Specifically, Kim et al. (24) showed, through in vivo experiments in the *Drosophila* embryo, that changing the level of one of the substrates of the MAPK cascade influences the level of MAPK phosphorylation. Additionally, Ventura et al. (23) showed, through experiments on a reconstituted covalent modification cycle, that the addition of a downstream target changes the steady-state value of the modified protein of the upstream cycle. These results are promising; however, additional experiments are required to validate the attenuation/amplification predictions of this article on the higher levels of a cascade. Specifically, validating the prediction that the perturbation on the total protein concentration is attenuated as it propagates upstream is particularly appealing, because it does not depend on the specific parameter values. Furthermore, it requires us to measure the total phosphorylated protein, which is a much easier task to accomplish than measuring the free phosphorylated protein. We plan to validate experimentally this prediction in our future work.

SUPPORTING MATERIAL

Additional information, equations, and eight figures are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(11)00231-1.

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