CAN THE CANCER FIGHT BE WON? (UN DAS, SECTION EDITOR)

Dynamics and Sensitivity of Signaling Pathways

Michael A. Kochen[1] · Steven S. Andrews[1] · H. Steven Wiley[2] · Song Feng[3] · Herbert M. Sauro[1]

Abstract

Purpose of Review Signaling pathways serve to communicate information about extracellular conditions into the cell, to both the nucleus and cytoplasmic processes to control cell responses. Genetic mutations in signaling network components are frequently associated with cancer and can result in cells acquiring an ability to divide and grow uncontrollably. Because signaling pathways play such a significant role in cancer initiation and advancement, their constituent proteins are attractive therapeutic targets. In this review, we discuss how signaling pathway modeling can assist with identifying effective drugs for treating diseases, such as cancer. An achievement that would facilitate the use of such models is their ability to identify controlling biochemical parameters in signaling pathways, such as molecular abundances and chemical reaction rates, because this would help determine effective points of attack by therapeutics.

Recent Findings We summarize the current state of understanding the sensitivity of phosphorylation cycles with and without sequestration. We also describe some basic properties of regulatory motifs including feedback and feedforward regulation.

Summary Although much recent work has focused on understanding the dynamics and particularly the sensitivity of signaling networks in eukaryotic systems, there is still an urgent need to build more scalable models of signaling networks that can appropriately represent their complexity across different cell types and tumors.

Keywords Dynamics · Sensitivity · Signaling networks · Cancer

✉ Michael A. Kochen
kochenma@uw.edu

Steven S. Andrews
steven.s.andrews@gmail.com

H. Steven Wiley
Steven.Wiley@pnnl.gov

Song Feng
song.feng@pnnl.gov

Herbert M. Sauro
hsauro@uw.edu

[1] Department of Bioengineering, University of Washington, Seattle, WA, USA

[2] Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, WA, USA

[3] Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA, USA

Introduction

Cellular signaling pathways serve to communicate information about extracellular conditions into the cell, to both the nucleus and cytoplasmic processes to control cell responses. These pathways also engage in various types of signal processing, such as integrating signals over time [1], converting graded signals to switch-like ones [2], and converting signal strength to signal duration [3]. In eukaryotes, these pathways tend to be highly interconnected, encompassing cross-talk and signal processing between multiple pathways. Genetic mutations in signaling network components are frequently associated with cancer and can result in cells acquiring the ability to divide and grow uncontrollably. A number of important signaling pathways have been identified as frequently genetically altered in cancers. These include the RTK/RAS/MAP kinase pathway, PI3K/Akt signaling, WNT signaling and many others. It has been reported that 46% of cancers are associated with alterations in the RTK/RAS/MAP kinase signaling network [4]. Because signaling pathways play such a significant role in cancer initiation and advancement, signaling pathways offer an attractive therapeutic target.

Conventional targeted drug discovery, in which a drug is designed to inhibit a specific gene product in a specific signaling pathway, has rarely led to substantial therapeutic effects for complex diseases such as cancer [5]. These failures arise from poor target specificity, undesirable side effects, and acquired resistance, many of which can be traced to an inadequate understanding of the drug's impact on the overall signaling pathway. Quantitative pathway modeling offers a partial solution to these problems because it can provide a deeper understanding of the impact on the interconnections of the cellular systems being targeted. It can show how a drug affects an entire signaling pathway and cross-talk to other pathways. It can also show how sensitive a pathway's output is to many possible perturbations, which can help identify drug targets as well as synergistic multi-target treatments.

As an example, several antibacterial drugs have been developed to target the LpxC protein in *E. coli*, which is a natural control point in the metabolism of lipopolysaccharide, an essential cell wall component. However, progress has been hampered by rapid evolution of pathogen resistance [6]. Subsequent modeling [7] showed that feedback in this system maintains constant flux through this control point, which reduces drug effectiveness and offers multiple routes for resistance. This modeling also identified other targets that are likely to be more effective.

This raises the question of whether signaling pathway modeling can assist with identifying effective drugs for treating diseases, such as cancer. An achievement that would facilitate this goal would be identifying sensitive biochemical parameters in signaling pathways, such as molecule abundances and chemical reaction rates. Such information would help determine effective points of attack by therapeutics. If a quantitative model includes a relevant target phenotype, it could be used to identify the most effective points where drugs should act.

In this review we describe work that has been done in recent years in understanding the dynamics and sensitivity of signaling networks that are found in eukaryotic systems. Unfortunately the literature is not extensive and is a topic that has been somewhat neglected by the community. We therefore review the current state of the field together with possible future challenges and areas that need more attention.

Basic Concepts

Signaling pathways exist at a wide range of complexities. Bacterial signaling is dominated by two-component regulatory systems, in which receptors phosphorylate and dephosphorylate downstream response regulators, and those regulators then transmit the signal to the necessary targets. However, these types of systems are rare in eukaryotes, being replaced by more complex signaling systems such as multi-step kinase cascades with dual phosphorylation steps. Indeed, a large fraction of signaling networks contain phosphorylation/dephosphorylation cycles. The phosphorylation step is often ATP dependent and is catalyzed by a kinase. The dephosphorylation step is a simple release of free phosphate catalyzed by a phosphatase.

Figure 1 shows a typical cycle where the ATP dependence and loss of phosphate have been omitted for clarity. In the remainder of the article we will designate A to represent unphosphorylated protein and AP phosphorylated protein. An
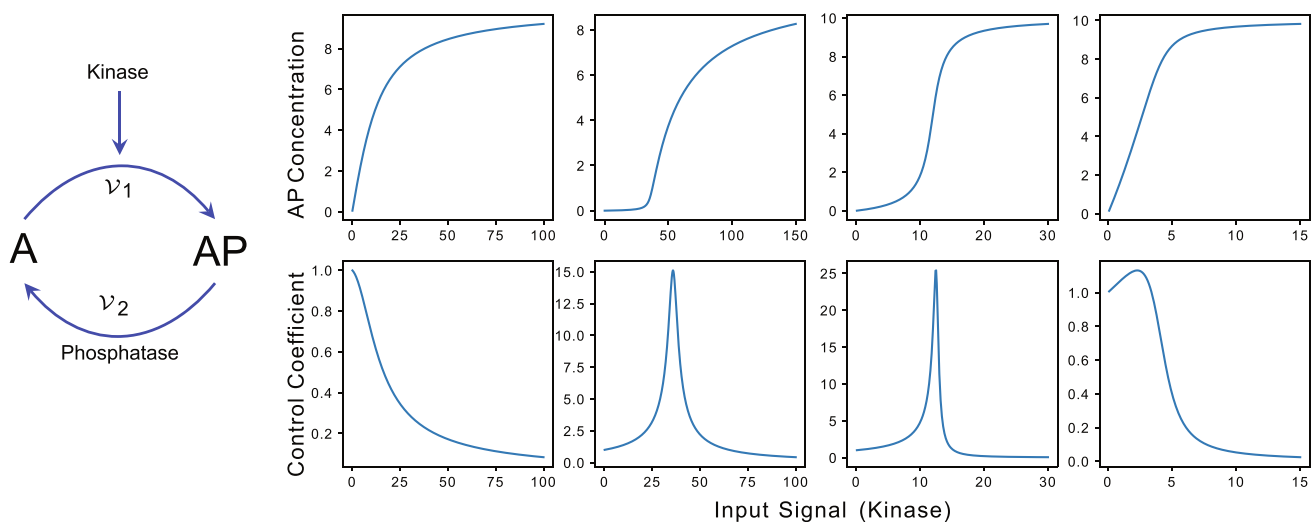

Fig. 1 Four regimes [8] of behavior for a simple phosphorylation cycle (left) where $A + AP = T$, v_1 is the forward rate catalyzed by a kinase and v_2 the reverse rate catalyzed by a phosphatase. The x-axis represent changes in kinase activity. **Top Panel:** Hyperbolic (Both Michaelis-Menten constants, K_m are above A and AP concentrations), Threshold Hyperbolic (Kinase K_m above A, phosphatase K_m below AP), Sigmoid (Both K_ms is below A and AP concentrations) and Linear (Kinase K_m below A, phosphatase K_m above AP). **Bottom panel:** Scaled sensitivity of the species AP with respect to change in the kinase. Code available: See end of article

important property of phosphorylation cycles emerges if we assume that protein synthesis and degradation rates are small compared to the phosphorylation and dephosphorylation rates. Under these conditions, the total mass of the cycle is fixed, that is $A + AP =$ constant $= T$. The symbol T will be used to indicate the total mass in a given cycle.

The steady-state properties of such cycles are well known [9]. For example, increasing kinase concentrations shift the steady state toward AP, and vice versa for phosphatase concentrations. AP can respond to kinase concentration changes with hyperbolic, sigmoidal, or linear behavior. Hyperbolic behavior occurs when the A and AP substrate concentrations are below the K_m values for the kinase and phosphatase enzymes, respectively, which can arise from low substrate concentrations, high enzyme concentrations, or fast dissociation of the enzyme-substrate complex. When enzyme saturation values are low, reaction rates are proportional to their substrate concentrations. Hyperbolic behavior also occurs even if the kinase reaction is saturated [8]. Sigmoidal behavior occurs when both substrate concentrations are above their respective K_m values and enzyme saturation occurs. In the limit of high saturation, both reaction rates are independent of their substrate concentrations, so the system shifts to being entirely A or entirely AP, depending on which reaction is faster. Finally, linear behavior arises if the phosphatase reaction is saturated but not the kinase reaction.

Metabolic control analysis [10] can help clarify these behaviors. Here, the elasticity of a reaction, $\varepsilon_{substrate}^{reaction}$, represents the variation of the reaction rate with respect to its substrate concentration, with the rough interpretation that it is the percent reaction rate increase upon a 1% substrate concentration increase. The elasticities for the kinase and phosphatase reactions are given by

$$\varepsilon 1A = \frac{\partial v_1}{\partial A} \frac{A}{v_1}, \tag{1}$$

$$\varepsilon 2AP = \frac{\partial v_2}{\partial AP} \frac{AP}{v_2}. \tag{2}$$

For an irreversible Michaelis-Menten mechanism, elasticities range from 0 for fully saturated enzymes to 1 for unsaturated enzymes. The control coefficient, $C_{enzyme}^{substrate}$, represents the variation of a substrate concentration with respect to an enzyme concentration. For a phosphorylation cycle, the sensitivity of the phosphorylated form AP with respect to the kinase activity has been shown to be [11, 12, 13]

$$C_k^{AP} = \frac{M_A}{M_{AP}\varepsilon 1A + M_A \varepsilon 2AP}, \tag{3}$$

where ε are the elasticities, M, the mole fraction and C_k^{AP}, the control coefficient of AP with respect to the kinase concentration. When the concentration of A and AP are

above the K_ms of the kinase and phosphatase, the elasticities will be small in magnitude. This results in a large sensitivity and corresponds to the case when the cycle is operating in the sigmoid regime. In engineering terminology, the coefficient $C_{e_1}^{AP}$, can be equated to the gain of the cycle between the kinase and output. For example, if both elasticities equal 0.1, and 90% of the mass is in the form of A, then the gain will equal 9, implying that a one percent increase in kinase activity will lead to a 9% increase in the phosphorylated form AP. This kind of increased sensitivity is called zero-order ultrasensitivity [14].

Cascades of phosphorylation cycles, where the output of one cycle is the kinase for the next cycle, are observed in nature leading to a compounding of the overall gain [15]. The pathway gain increases as a simple product of the separate cycle gains [16, 11]. For example, if a cascade is made up of three cycles and each cycle shows a gain of 9, then the overall gain of the cascade will be 729. It is clear that substantial amplification can be observed when combining cycles into layers.

Kholodenko et al. [11] showed that if we ignore sequestration, it is possible to define a unit amplification term r_j^i, where for a cascade of n layers, the overall sensitivity is given by

$$R_s^{p_n} = r_s^{p_1} r_{p_1}^{p_2} r_{p_2}^{p_3} ... r_{p_{n-1}}^{p_n}, \tag{4}$$

where r_j^i is defined the response given in Eq. (3) multiplied by the input elasticity from an earlier state stage: $\varepsilon_{p_1}^v$. Once there is sequestration between layers, the simple relationship (Eq. (4)) no longer holds [17•].

In addition to simple cycles, we also find doubly phosphorylated cycles in signaling pathways, particularly in the case of mitogen-activated protein kinase (MAPK) cascades. Figure 2 illustrates a double cycle. In doubly phosphorylated cycles, it is possible to achieve gains greater than one without the concentrations of the proteins going above the K_m [15]. However, the gain under these circumstances is limited to the number of phosphorylation sites (and can also be substantially less [18]). This kind of increased sensitivity is called first-order sensitivity because it arises when there is no saturation of the kinases or phosphatases. It is also possible to combine zero-order together with first-order to generate even higher gains [19•].

In terms of drug action, one can imagine at least two scenarios on phosphorylation cycles. A drug could bind irreversibly to either A or AP thus reducing the total mass, T, of the cycle; or a drug may bind reversibly to A or AP resulting in competition between the drug and binding of the kinase. These two cases are shown in Fig. 3
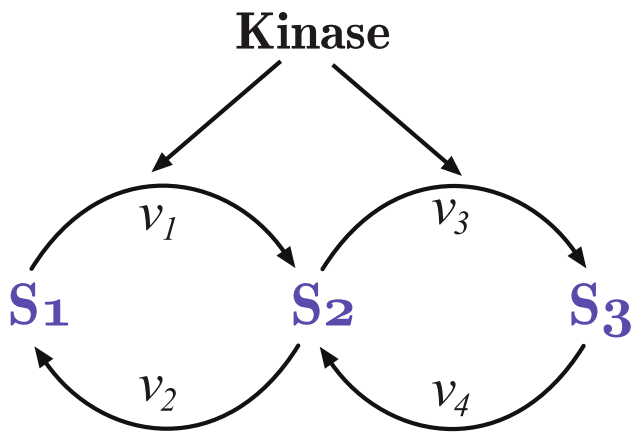
Kinase

Fig. 2 Double phosphorylation cycle

Investigating the sensitivity of a phosphorylation cycle to changes in T is straight forward with [20, 21]

$$C_T^{AP} = \frac{\varepsilon_1}{M_A \varepsilon_1 + M_{AP} \varepsilon_2}. \tag{5}$$

If the kinase and phosphatase are unsaturated then the elasticities will be roughly one. This means that C_T^{AP} will also be roughly equal to one. That is, when an irreversible inhibitor acts at a particular phosphorylation cycle it has a proportional effect on the output of the cycle. However, if the cycle is part of a layer of cycles, then this inhibition will be transmitted to downstream cycles and potentially amplified.

There have not been studies to date on the effect of reversible inhibition (left panel 3) on signaling cascades.

Feedback and Feedforward

Negative feedback loops are common components in signaling systems. In many cases, negative feedback loops are caused by activated downstream proteins inhibiting the activity of upstream proteins. Examples are seen in the Wnt [22], EGFR-Erk [23], and TGF-β [24, 25] pathways.

However, negative feedback can also occur through signal propagation back up the main signaling pathway due to either product inhibition [26] arising from reaction reversibility, or enzyme sequestration [27, 28•]. Negative feedback always reduces the signaling network gain, which is presumably undesirable in many cases. However, this cost is offset by the numerous control opportunities that negative feedback provides.

First, negative feedback is widely used in both engineered and biological systems to maintain specific system parameters at near-constant levels, independent of external perturbations and with reduced sensitivity to noise [29, 30, 31]. Biochemical mechanisms for this can be quite simple, such as a downstream protein that inhibits an upstream enzyme (Fig. 4A). As an example, the glycolysis metabolic pathway maintains constant intracellular ATP concentrations by using negative feedback to control the flux through the phosphofructokinase enzyme [31], where flux is reduced if ATP concentrations are too high and increased if ATP concentrations are too low. This type of feedback is relatively insensitive to its specific parameters. However, the particular value of that steady-state output does depend on the parameters. Negative feedback takes time to reach a new steady state when the network is perturbed. For example, if the input value is at one constant value and is then stepped up to a higher value, this increase propagates through the system to create an increased output. The negative feedback then takes effect, bringing the output value back down again to adapt the system to the new input value.

More precise adaptation can be achieved through integral control negative feedback, in which the difference between the system's input and output is integrated over time, and the value of that integral is then fed back to reduce the output. This type of control can produce "perfect adaptation," meaning that after the input changes, the output adapts exactly back to its previous level. Integral control was first recognized in the *E. coli* chemotaxis pathway [1] and has also been found in the yeast osmoregulation [32] system. A particularly simple implementation of integral feedback control is through "antithetic integral control," in which the system generates molecules at both its input and

Fig. 3 Two possible modes of inhibitor action. *D* represents the inhibitor. Binding of inhibitor can be irreversible or reversible
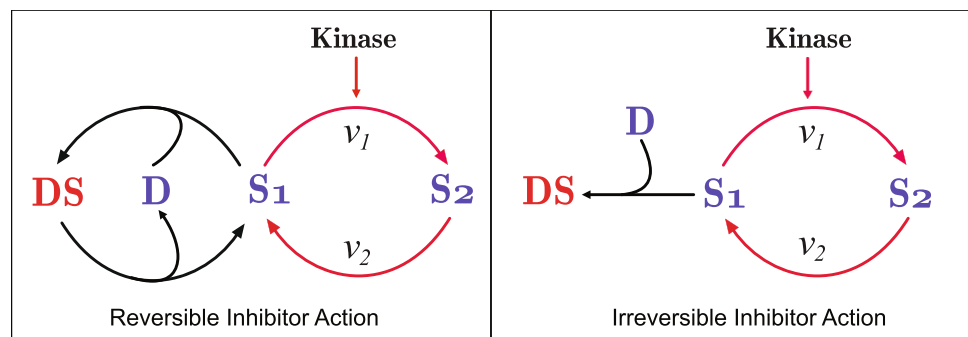
Fig. 4 Negative feedback applications. (**A**) Simple negative feedback to an enzyme to keep concentrations of X_2 to X_n and the output nearly constant. (**B**) Antithetic integral control, which creates perfect adaptation of the output. (**C**) Feedback for linear signaling from input to output. (**D**) A specific example of linear signaling using negative feedback, here in the Ras-ERK pathway
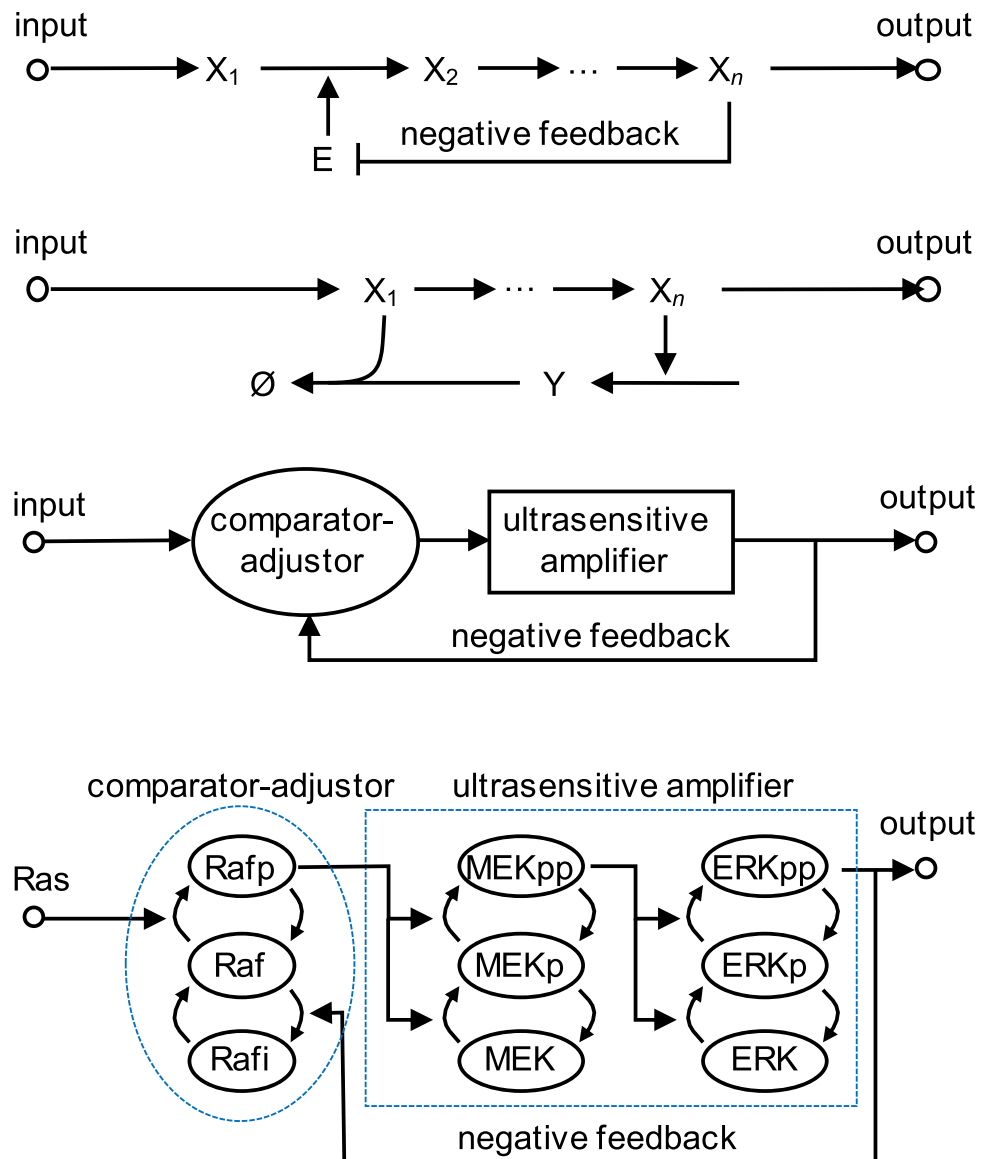
output, each at constant rates, and these molecules bind tightly to each other (Fig. 4B). The remaining unbound molecules represent the integral of the difference between input and output.

A separate role of negative feedback is to linearize the outputs of signaling systems, making them directly proportional to their inputs. This use has been well known in electronics for almost a century [33] but was only recently discovered to operate in biochemical networks [34, 35, 36, 37•]. Achieving linearity in biochemical networks is more challenging than it might appear because all signals asymptotically approach some maximum level, and most simple networks approach this level gradually, resulting in hyperbolic or sigmoidal response curves (see Fig. 1). Linear response curves are possible from single cycles as well, also shown in Fig. 1, but these cycles become non-linear when

linked in series. This is because when the intermediate enzymes are saturated for linear signaling, it sequesters them from the prior cycle, which makes that one non-linear [34]. Negative feedback can solve this biochemical linearity problem in the same way as in electronic systems. Here, the downstream signal is fed back to a "comparator-adjustor" that compares the output to the input and then sends the difference value to an ultrasensitive amplifier to generate the output (Fig. 4C). The Ras-ERK pathway uses this mechanism (Fig. 4D). Here, Ras activates Raf, which acts an a comparator-adjustor; Raf is phosphorylated (activated) by an input signal and phosphorylated (inactivated) by a negative feedback signal, with the result that the net concentration of activated Raf represents the difference between system input and output (Fig. 4D). This value is then amplified through an ultrasensitive kinase cascade [38, 36•].

Systems with negative feedback loops generally have a tendency to oscillate due to the feedback always acting in opposition to the output. Actual oscillations depend on the feedback strength, system gain, and damping forces within the system [29]. Damping forces in physical oscillators typically arise from energy dissipating influences, such as mechanical friction or electrical resistance. In biochemical oscillators, they often arise instead from the stochastic nature of the biochemical reaction, which removes phase coherence and hence damps oscillations; here, energy dissipation is required to *maintain* oscillations [39]. The configuration of some cell signaling networks can also dampen oscillations [40]. Biochemical oscillations in cells help control cell cycles and circadian rhythms, but there are cases where their purpose is less clear, including in glycolysis [41], the EGFR-ERK signaling system [42], and the NF-κB system [40]. Various roles for these oscillations have been proposed [41, 43], but they could also be, at least partially, simply a side effect of negative feedback that has another important regulatory role.

Positive feedback loops also connect elements from downstream parts of a network to upstream ones, but act to increase the downstream signal. Their steady-state impact is to increase the gain of a signaling pathway, meaning that they compress the dose-response curve. For example, the MAPK cascade in Xenopus oocytes includes a positive feedback loop that increases the Hill coefficient of the dose-response curve from 5 to 35, essentially creating an all-or-none response [44]. Even stronger positive feedback converts the dose-response curve from a steep sigmoid to an S-shaped curve containing a region of bistability [45, 46]. Here, there is a specific input range over which the signaling system output is stable with either high or low outputs. Moving outside of the stable range can flip the system between the two alternative states. Although bistability requires positive feedback, it does not have to be effected through an external circuit element. For example, enzyme saturation and competitive inhibition are sufficient in cycles that utilize dual phosphorylation mechanisms [19•]. Bistability is a necessary condition for relaxation oscillators. Additionally, if the system's bistable range covers all possible input parameters, then the output cannot be flipped between states, but is irreversibly locked into one state or the other. This behavior is useful for irreversible cell state changes, such as for one-way progression through the cell cycle, and irreversible cell fate decisions during differentiation. The lambda-phage lysis/lysogeny decision [47] is a particularly well-studied example of positive feedback being used to lock in a particular fate decision.

Feedforward elements in cell signaling transmit the signal downstream parallel to the main signaling pathway followed by signal recombination. Positive feedforward loops, in which the feedforward signal has the same sign as the main signal, are called coherent, while negative feedforward loops that have the opposite effect are called incoherent [48]. Feedforwards have been studied much less than feedbacks but share some of the same control behaviors. In particular, incoherent feedforward loops can perform perfect adaptation [49, 50] much like integral feedback. This was observed experimentally in a Ras signaling pathway used for *Dictyostelium discoideum* chemotaxis, where the signal was sent to two proteins simultaneously, with one activating a downstream Ras protein and the other inhibiting it [51].

A particularly interesting type of feedforward loop is a push-pull mechanism [34, 52•]. In a simple version (Fig. 5), there are two proteins, here called X and Y, each of which cycle between inactive and active states which are shown in the figure as unphosphorylated or phosphorylated states. The active copies of X phosphorylate Y, pushing it toward greater activity, as is typical in kinase cascades. Meanwhile, the inactive X dephosphorylates Y, pulling it back toward inactivity. Push-pull mechanisms are often described as paradoxical signaling components or incoherent feedforward loops [53, 54] because the same X protein acts to both activate and inactivate Y, but these descriptions are misleading because the behaviors arise from different states of the X protein; indeed, it would be more appropriate to call it a coherent feedforward loop due to the double sign reversal in the feedforward loop. The symmetry of the push-pull mechanism gives it several unique properties. It is the only type of feedback or feedforward that can transmit signals linearly when the relevant enzymes are unsaturated [34]. It also confers substantial robustness to variability in the system's components [55] and is able to provide ratiometric sensing
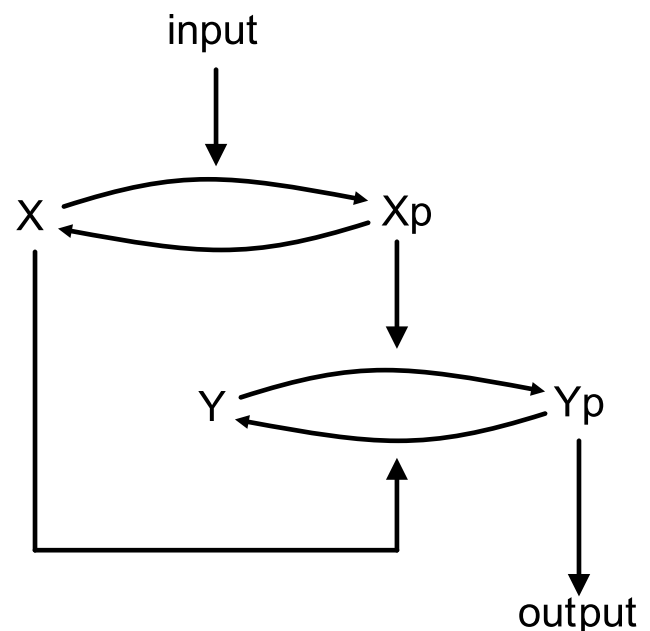

Fig. 5 Diagram of a push-pull mechanism

rather than absolute sensing, meaning that the activity of Y depends on the *fraction* rather than the amount of X that is active [56•].

Many push-pull mechanisms have been identified, including bacterial two-component signaling [57, 55], the yeast pheromone response receptors [56•], the yeast MAP kinase pathway [34], and the heat shock response in Xenopus oocytes [58].

Effects of Sequestration

Ultrasensitivity in signaling pathways takes the form of sharp sigmoidal responses to small changes in an input signal (Fig. 1) and allows for threshold-based switch-like behavior. With respect to phosphorylation/dephosphorylation, or any other covalent modification cycle, the 'speed' at which this switch-like transition happens, as the kinase level is increased, can be quantified with a response coefficient defined, as in [14], as

$$R_c = \frac{K/P \text{ at } 90\% \, AP}{K/P \text{ at } 10\% \, AP}, \quad (6)$$

where K/P is the ratio of the kinase and phosphatase enzymes and AP is phosphorylated form of the protein. An alternative approach to the response coefficient in Eq. (6) is one that makes use of metabolic control analysis as discussed above for Fig. 1 [13]. Note that in Eq. (3) there are no terms for enzyme-substrate complexes. The derivation of that equation assumes that the concentrations of the modified and unmodified substrates are much greater than the enzymes that target them. The amount of bound substrate is thus negligible, allowing enzyme-substrate complexes to be ignored.

However, enzymes and their targets often have comparable cellular concentrations [59, 17•] as in the MAPK cascade. Increasing enzyme concentrations to levels on par with their target substrates may lower the concentration of free substrates to levels below their K_m values [17•], a condition for ultrasensitivity. Substrate sequestration could also render them unavailable to other enzymes in the pathway. It is therefore necessary to consider the role of substrates sequestration in the overall behavior of signaling pathways.

Inclusion of enzyme-substrate complexes in mechanistic computational models [14] has demonstrated that increasing enzyme concentrations, and subsequently increasing the levels of enzyme-substrate complexes, results in a significant reduction in sensitivity as the ratio of enzyme to total substrate grows large. Another analysis approach is to extend the existing MCA-based sensitivity (Eq. (3)) to accommodate the additional enzyme-substrate complex components [17•]. The derived result is

$$C_k^{AP} = \frac{M_A}{M_{AP}\varepsilon 1A + M_A\varepsilon 2AP + M_{K*A}\varepsilon 1A\varepsilon 2AP + M_{P*AP}}, \quad (7)$$

where the new terms M_{K*A} and M_{P*AP} are the mole fractions of bound kinase and phosphatase respectively. The end result is twofold. If we assume the elasticities remain constant, the shift in mass of A to the denominator, and the shift in mass of AP to a term in the denominator lacking an elasticity multiplier, will lower sensitivity. It is also clear that if we have sufficient concentrations of enzymes such that we cannot disregard the enzyme-substrate complexes, sequestration will lower the concentrations of free substrate and result in higher elasticities. This in turn also lowers sensitivities.

Higher enzyme levels and substrate sequestration carry clear ramifications for signaling pathway dynamics. The four operating regimes shown in Fig. 1 consider the case in which the substrate concentrations are much greater than that of the enzymes. By allowing the enzyme concentrations to increase to levels on par with, or greater than, the substrate, the number of operating regimes greatly expands [60]. The four operating regimes in Fig. 1 have also been analyzed in the context of downstream loads on the system [61]. Substrates are often enzymes in their own right and can act on targets that alter upstream behavior of the system, a process termed retroactivity (Fig. 6) [27•]. These retroactivity effects induce five distinct transitions between the aforementioned operating regimes depending on the initial regime and the substrate (phosphorylated or unphosphorylated) taking part in downstream interactions.

Retroactivity brought about via downstream substrate sequestration is a key behavioral consideration in covalent modification-based signaling pathways [62, 63, 64] like the MAPK cascade. This was demonstrated in [28] with a computational model of a three-level cascade of phosphorylation cycles that incorporated the effects of enzyme-substrate complexes. This showed that signal responses for the two intermediate cycles had lower maximum steady-state values of the active substrate as compared to the final cycle that had no downstream targets. This implies there is sequestration of the intermediate active substrates from their role as enzymes by downstream targets. There was also a reduction in sensitivity when compared to a model that doesn't include the effects of sequestration [14]. Retroactivity has also been demonstrated experimentally, for example, in [65] with a uridylylation/deuridylylation cycle derived from *E. coli*. It was shown that in the presence of a downstream binding target (NRII) for the substrate (PII) the sensitivity to an increase in a regulator (glutamine) was diminished in comparison to the case lacking a downstream target. It has also been shown

Fig. 6 Retroactivity in sequential phosphorylation cycles. The phosphorylated species in the upstream cycle (X_p) acts as a kinase in the downstream cycle (red arrow). This interaction induces indirect upstream effects (dashed gray arrow) on the upstream cycle by sequestration of the substrate for the upstream phosphatase
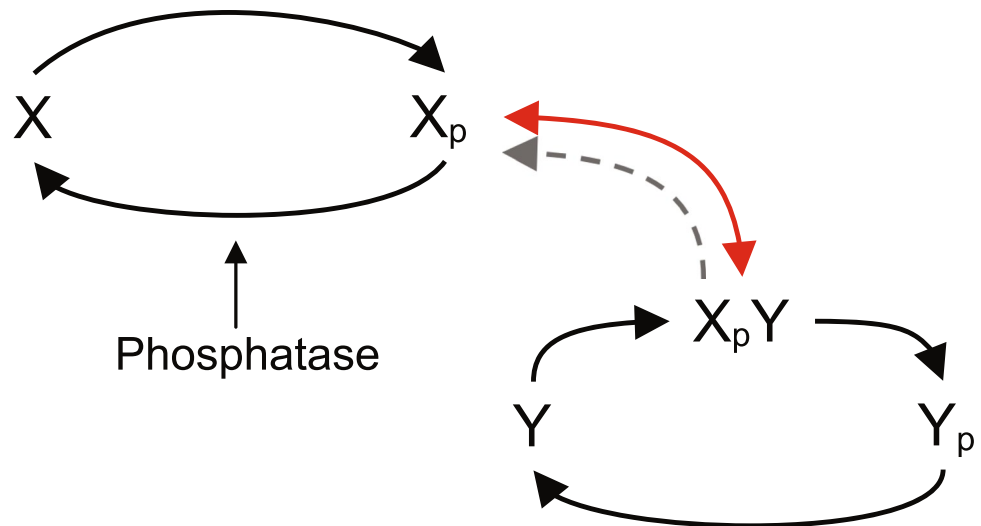
that substrates of phosphorylated MAPK, the last phosphorylation cycle in the MAPK cascade, inhibited subsequent MAPK dephosphorylation [66].

Substrate sequestration and its potential roles in system dynamics have been explored, computationally, in the context of various mechanistic models. For example, phosphatase sequestration in the presence of an opposing fast, low affinity kinase can cause signal desensitization, while sequestration in the context of a cycle with a doubly phosphorylated substrate can result in a signal with a sign-sensitive delay [17•]. These two scenarios potentially obviate the need for negative feedback and coherent feedforward loops respectively. Substrate sequestration combined with multiple substrate phosphorylation sites can also be used to design modules with optimal threshold and ultrasensitive responses as detailed in [67]. Under the right conditions ultrasensitivity in a phosphorylation cycle may be achieved even when the enzymes are unsaturated. As demonstrated in [68], when the kinase and phosphatase require docking to a separate site before catalysis the docking site can serve as a means for the enzyme to sequester the substrate from the opposing enzyme. For example a kinase binding to a fully phosphorylated substrate could block the opposing phosphatase. In this way, sequestering of the substrate would enable ultrasensitivity rather than destroying it.

Bistability in double phosphorylation cycles (Fig. 2) emerges from the saturation of at least one of the enzymes by their fully phosphorylated/dephosphorylated substrate [19•]. However, accounting for substrate sequestration by incorporating the complexes into the equations produced a reduced parameter domain (e.g., enzyme K_m values) on which bistability is possible. In addition, when one enzyme is left unsaturated the bistability domain can

shrink considerably. Sequestration also has a deleterious effect on the stability of steady states in cascades of double phosphorylation cycles [69] since stability is negatively correlated to high levels of enzyme bound substrate in the last cycle of the cascade. The bistability conditions laid out in [19•] were subsequently combined with negative feedback loops [70] to construct relaxation and ring oscillators, while work in [71] further showed that oscillations are achievable with just a double phosphorylation cycle (Fig. 2) within certain parameter ranges and, in general, an excess of substrate over enzyme. Nevertheless, increasing enzyme concentrations and the subsequent substrate sequestration are known to eliminate oscillatory behavior, as was demonstrated in [17•] using an oscillatory model of the MAPK cascade along with a negative feedback loop.

The majority of the work on substrate sequestration has been computational, and thus hypothetical. In this section we have discussed some of the more important aspects of sequestration as well as a few of its potential roles. Additional computational studies with a broader perspective on the topic can be found in [72, 73, 74, 75]. A limited number of cases for zero-order sensitivity have been established experimentally, for which sequestration could potentially play a role, including the phosphorylation cycles of glycogen phosphorylase [76] and isocitrate dehydrogenase [77]. Of course the general concept of sequestration extends much more broadly than substrate sequestration by enzymes in covalent modification cycle. Examples include the hypothesis that the bistability in the Raf-Mek-Erk signal cascade is governed by the sequestration of Mek by Erk resulting in a positive feedback loop [78] and the sequestration of enzymes via pathway competition as demonstrated with a model system of the Jak kinase [79].

Discussion

In this review, we described how the intrinsic properties of enzymes impact the sensitivity and dynamics of signaling networks. This knowledge is essential in predicting how drugs and genetic perturbations will impact those networks. Knowledge of how these networks operate as an integrated system, however, is very incomplete. For example, we have a reasonably good understanding of the enzymology of the three-component Raf-Mek-Erk cascade (Fig. 4), but the upstream and downstream components are far less understood. Because substrate sequestration can have a profound impact on the function of signaling networks, this lack of contextual understanding of different components of signaling networks limits our ability to build predictive models of their dynamic behavior. There is also a lack of information on the relevant concentrations and distributions of signaling pathway enzymes and substrates in cells. Traditional methods for parameterization of signaling models usually generate "lumped" parameters in which the concentrations of both enzymes and substrates conflated with rate constants [80]. This can be useful for modeling the behavior of specific cell system, but such models generally cannot predict the impact of changing enzyme and protein concentrations outside of a limited range. This limits their usefulness for predicting the impact of drugs or genetic changes, such as copy number variations. Several recent studies, however, have quantified pathway protein abundance as explicit model parameters and have shown that this improves the ability of models to predict the impact of both drugs and altered protein abundance [81]. As the technologies needed to quantify the often-low levels of signaling proteins improve, inclusion of their abundance values in models should improve their predictive power.

A further difficulty in building predictive models of signaling networks is their complexity and interconnectedness. The above examples of motifs and network topologies represent only a small part of signaling networks in cells. Actual signaling networks comprise a complex set of recursive, interconnected pathways that link intercellular information processing with changing extracellular conditions. As outside conditions change, these networks adapt dynamically such that the relative level of their constituents is always in flux. Dealing with this continual rewiring is a challenge that will require new approaches in mathematically representing signaling pathways.

One approach is to represent subsections of these networks as "modules" that explicitly include their dynamic response potential [82]. Signaling networks are widely thought to be modular due to both the need for evolutionary flexibility in reconfiguring networks in response to selective pressure [83] and as a mechanism to reduce the impact of biological noise [84]. An inherent aspect of these modules is that their interaction with other parts of the network should be restricted to just a few "interface" proteins that insulate the modules from other parts of the networks. This insulation can be achieved through negative feedback or low substrate sequestration [27•]. Thus, understanding the dynamics between different components of signaling pathways is crucial to understanding their overall regulatory architecture.

Learning to identify and build models of the constituent modules that comprise signaling networks will provide a powerful new approach for building scalable models of signal transduction. However, understanding the impact on drugs on these networks will always require a fine level understanding of how they impact specific biochemical reactions. Thus, an appreciation of these networks in terms of their underlying biochemistry is crucial in building realistic, and ultimately predictive models of their function in both health and disease.

Code Availability Code for generating Fig. 1 is available at https://github.com/sys-bio/CodeForPublishedPapers/tree/main/CurrentPathobiologyReports2021.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Papers of particular interest, published recently, have been highlighted as:
● Of importance
●● Of major importance

1. Yi TM, Huang Y, Simon MI, Doyle J. Robust perfect adaptation in bacterial chemotaxis through integral feedback control. Proceedings of the National Academy of Sciences. 2000;97(9):4649–53.
2. Ferrell JE Jr, Ha SH. Ultrasensitivity part iii: cascades, bistable switches, and oscillators. Trends in Biochemical Sciences. 2014;39(12):612–8.
3. Behar M, Hao N, Dohlman HG, Elston TC. Dose-to-duration encoding and signaling beyond saturation in intracellular signaling networks. PLoS Comput Biol. 2008;4(10):e1000197.

4. Sanchez-Vega F, Mina M, Armenia J, Chatila WK, Luna A, La KC, Dimitriadoy S, Liu DL, Kantheti HS, Saghafinia S, et al. Oncogenic signaling pathways in the cancer genome atlas. Cell. 2018;173(2):321–37.

5. Aggarwal BB, Sethi G, Baladandayuthapani V, Krishnan S, Shishodia S. Targeting cell signaling pathways for drug discovery: an old lock needs a new key. Journal of Cellular Biochemistry. 2007;102(3):580–92.

6. Walsh CT, Wencewicz TA. Prospects for new antibiotics: a molecule-centered perspective. The Journal of Antibiotics. 2014;67(1):7–22.

7. Emiola A, George J, Andrews SS. A complete pathway model for lipid a biosynthesis in escherichia coli. PloS One. 2015;10(4):e0121216.

8. Gomez-Uribe C, Verghese GC, Mirny LA. Operating regimes of signaling cycles: statics, dynamics, and noise filtering. PLoS Comput Biol. 2007;3(12):e246.

9. Stadtman E, Chock P. Superiority of interconvertible enzyme cascades in metabolic regulation: analysis of monocyclic systems. Proceedings of the National Academy of Sciences. 1977;74(7):2761–5.

10. Fell DA. Metabolic control analysis: a survey of its theoretical and experimental development. Biochemical Journal. 1992;286(2):313–30.

11. Kholodenko BN, Hoek JB, Westerhoff HV, Brown GC. Quantification of information transfer via cellular signal transduction pathways. FEBS Letters. 1997;414(2):430–4.

12. Sauro HM, Kholodenko BN. Quantitative analysis of signaling networks. Progress in Biophysics and Molecular Biology. 2004;86(1):5–43.

13. Small JR, Fell DA. Covalent modification and metabolic control analysis: Modification to the theorems and their application to metabolic systems containing covalently modifiable enzymes. European Journal of Biochemistry. 1990;191(2):405–11.

14. Goldbeter A, Koshland DE. An amplified sensitivity arising from covalent modification in biological systems. Proceedings of the National Academy of Sciences. 1981;78(11):6840–4.

15. Huang CY, Ferrell JE. Ultrasensitivity in the mitogen-activated protein kinase cascade. Proceedings of the National Academy of Sciences. 1996;93(19):10078–83.

16. Brown GC, Hoek JB, Kholodenko BN. Why do protein kinase cascades have more than one level? Trends in Biochemical Sciences. 1997;22(8):288–288.

17.• Blüthgen, N., Bruggeman, F.J., Legewie, S., Herzel, H., Westerhoff, H.V., Kholodenko, B.N.: Effects of sequestration on signal transduction cascades. The FEBS Journal 273(5), 895–906 (2006). This paper demonstrates the effects of increasing enzyme concentration, and subsequent substrate sequestration, on zero-order ultrasensitivity.

18. Gunawardena J. Multisite protein phosphorylation makes a good threshold but can be a poor switch. Proceedings of the National Academy of Sciences. 2005;102(41):14617–22.

19.• Markevich, N.I., Hoek, J.B., Kholodenko, B.N.: Signaling switches and bistability arising from multisite phosphorylation in protein kinase cascades. The Journal of Cell Biology 164(3), 353–359 (2004). This paper was the first to show that sequestration could have major implications on the qualitative behaviour of signaling networks.

20. Sauro HM. Moiety-conserved cycles and metabolic control analysis: problems in sequestration and metabolic channelling. BioSystems. 1994;33(1):55–67.

21. Sauro, H.M.: Systems biology: an introduction to metabolic control analysis. Ambrosius Publishing (2018)

22. Lustig B, Jerchow B, Sachs M, Weiler S, Pietsch T, Karsten U, van de Wetering M, Clevers H, Schlag PM, Birchmeier W, et al.

Negative feedback loop of wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. Molecular and Cellular Biology. 2002;22(4):1184–93.

23. Sturm OE, Orton R, Grindlay J, Birtwistle M, Vyshemirsky V, Gilbert D, Calder M, Pitt A, Kholodenko B, Kolch W. The mammalian mapk/erk pathway exhibits properties of a negative feedback amplifier. Science Signaling. 2010;3(153):ra90–ra90.

24. Miyazono K. Positive and negative regulation of tgf-beta signaling. Journal of Cell Science. 2000;113(7):1101–9.

25. Stroschein SL, Wang W, Zhou S, Zhou Q, Luo K. Negative feedback regulation of tgf-β signaling by the snon oncoprotein. Science. 1999;286(5440):771–4.

26. Cornish-Bowden A, Cárdenas ML. Information transfer in metabolic pathways: effects of irreversible steps in computer models. European Journal of Biochemistry. 2001;268(24):6616–24.

27.• Del Vecchio, D., Ninfa, A.J., Sontag, E.D.: Modular cell biology: retroactivity and insulation. Molecular Systems Biology 4(1), 161 (2008). This paper demonstrated the effects of retroactivity on upstream components and introduced an amplifier based compensatory mechanism.

28. Ventura AC, Sepulchre JA, Merajver SD. A hidden feedback in signaling cascades is revealed. PLoS Comput Biol. 2008;4(3):e1000041.

29. Bechhoefer J. Feedback for physicists: A tutorial essay on control. Reviews of Modern Physics. 2005;77(3):783.

30. Rao CV, Wolf DM, Arkin AP. Control, exploitation and tolerance of intracellular noise. Nature. 2002;420(6912):231–7.

31. Sauro HM. Control and regulation of pathways via negative feedback. Journal of The Royal Society Interface. 2017;14(127):20160848.

32. Muzzey D, Gómez-Uribe CA, Mettetal JT, van Oudenaarden A. A systems-level analysis of perfect adaptation in yeast osmoregulation. Cell. 2009;138(1):160–71.

33. Black HS. Stabilized feedback amplifiers. Bell System Technical Journal. 1934;13(1):1–18.

34. Andrews SS, Peria WJ, Richard CY, Colman-Lerner A, Brent R. Push-pull and feedback mechanisms can align signaling system outputs with inputs. Cell Systems. 2016;3(5):444–55.

35. Nevozhay D, Adams RM, Murphy KF, Josić K, Balázsi G. Negative autoregulation linearizes the dose-response and suppresses the heterogeneity of gene expression. Proceedings of the National Academy of Sciences. 2009;106(13):5123–8.

36.• Nunns, H., Goentoro, L.: Signaling pathways as linear transmitters. Elife. 2018; 7, e33617. The authors investigated the mechanisms that three different biological pathways use to produce linear signaling

37. Sauro, H.M., Ingalls, B.: Mapk cascades as feedback amplifiers. arXiv (2007)

38. Andrews SS, Brent R, Balázsi G. Signaling systems: Transferring information without distortion. Elife. 2018;7:e41894.

39. Cao Y, Wang H, Ouyang Q, Tu Y. The free-energy cost of accurate biochemical oscillations. Nature Physics. 2015;11(9):772–8.

40. Hoffmann A, Levchenko A, Scott ML, Baltimore D. The iκb-nf-κb signaling module: temporal control and selective gene activation. Science. 2002;298(5596):1241–5.

41. Boiteux A, Goldbeter A, Hess B. Control of oscillating glycolysis of yeast by stochastic, periodic, and steady source of substrate: A model and experimental study. Proc Nat Acad Sci USA. 1975;72(10):3829–33.

42. Shankaran H, Ippolito DL, Chrisler WB, Resat H, Bollinger N, Opresko LK, Wiley HS. Rapid and sustained nuclear-cytoplasmic erk oscillations induced by epidermal growth factor. Molecular Systems Biology. 2009;5(1):332.

43. Shankaran H, Wiley HS. Oscillatory dynamics of the extracellular signal-regulated kinase pathway. Current Opinion in Genetics & Development. 2010;20(6):650–5.

44. Ferrell JE, Machleder EM. The biochemical basis of an all-or-none cell fate switch in xenopus oocytes. Science. 1998;280(5365):895–8.

45. Ferrell JE Jr. Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability. Current Opinion in Cell Biology. 2002;14(2):140–8.

46. Xiong W, Ferrell JE. A positive-feedback-based bistable 'memory module'that governs a cell fate decision. Nature. 2003;426(6965):460–5.

47. Arkin A, Ross J, McAdams HH. Stochastic kinetic analysis of developmental pathway bifurcation in phage λ-infected escherichia coli cells. Genetics. 1998;149(4):1633–48.

48. Shen-Orr SS, Milo R, Mangan S, Alon U. Network motifs in the transcriptional regulation network of escherichia coli. Nature Genetics. 2002;31(1):64–8.

49. Ma W, Trusina A, El-Samad H, Lim WA, Tang C. Defining network topologies that can achieve biochemical adaptation. Cell. 2009;138(4):760–73.

50. Sontag ED. Remarks on feedforward circuits, adaptation, and pulse memory. IET Systems Biology. 2009;4(1):39–51.

51. Takeda K, Shao D, Adler M, Charest PG, Loomis WF, Levine H, Groisman A, Rappel WJ, Firtel RA. Incoherent feedforward control governs adaptation of activated ras in a eukaryotic chemotaxis pathway. Science Signaling. 2012;5(205):ra2–ra2.

52.• Chock, P., Stadtman, E.: Superiority of interconvertible enzyme cascades in metabolic regulation: analysis of multicyclic systems. Proceedings of the National Academy of Sciences 74(7), 2766–2770 (1977). This paper is of historical importance because it was one of the first to consider phosphorylation cycles from a computational perspective.

53. Hart Y, Alon U. The utility of paradoxical components in biological circuits. Molecular Cell. 2013;49(2):213–21.

54. Rangamani P, Levy MG, Khan S, Oster G. Paradoxical signaling regulates structural plasticity in dendritic spines. Proceedings of the National Academy of Sciences. 2016;113(36):E5298–307.

55. Shinar G, Milo R, Martínez MR, Alon U. Input-output robustness in simple bacterial signaling systems. Proceedings of the National Academy of Sciences. 2007;104(50):19931–5.

56.• Bush, A., Vasen, G., Constantinou, A., Dunayevich, P., Patop, I.L., Blaustein, M., Colman-Lerner, A.: Yeast gpcr signaling reflects the fraction of occupied receptors, not the number. Molecular Systems Biology 12(12), 898 (2016) This paper shows that yeast cells use a push-pull mechanism to transmit the fractional receptor activity rather than absolute activity.

57. Batchelor E, Goulian M. Robustness and the cycle of phosphorylation and dephosphorylation in a two-component regulatory system. Proceedings of the National Academy of Sciences. 2003;100(2):691–6.

58. Conde R, Belak ZR, Nair M, O'Carroll RF, Ovsenek N. Modulation of hsf1 activity by novobiocin and geldanamycin. Biochemistry and Cell Biology. 2009;87(6):845–51.

59. Albe KR, Butler MH, Wright BE. Cellular concentrations of enzymes and their substrates. Journal of Theoretical Biology. 1990;143(2):163–95.

60. Straube R. Operating regimes of covalent modification cycles at high enzyme concentrations. Journal of Theoretical Biology. 2017;431:39–48.

61. Parundekar, A., Viswanathan, G.A.: Retroactivity induced operating regime transition in a phosphorylation-dephosphorylation cycle. bioRxiv (2020)

62. Ossareh HR, Ventura AC, Merajver SD, Del Vecchio D. Long signaling cascades tend to attenuate retroactivity. Biophysical Journal. 2011;100(7):1617–26.

63. Sepulchre JA, Merajver SD, Ventura AC. Retroactive signaling in short signaling pathways. PloS One. 2012;7(7):e40806.

64. Wynn ML, Ventura AC, Sepulchre JA, García HJ, Merajver SD. Kinase inhibitors can produce off-target effects and activate linked pathways by retroactivity. BMC Systems Biology. 2011;5(1):1–15.

65. Ventura AC, Jiang P, Van Wassenhove L, Del Vecchio D, Merajver SD, Ninfa AJ. Signaling properties of a covalent modification cycle are altered by a downstream target. Proceedings of the National Academy of Sciences. 2010;107(22):10032–7.

66. Kim Y, Paroush Z, Nairz K, Hafen E, Jiménez G, Shvartsman SY. Substrate-dependent control of mapk phosphorylation in vivo. Molecular Systems Biology. 2011;7(1):467.

67. Liu X, Bardwell L, Nie Q. A combination of multisite phosphorylation and substrate sequestration produces switchlike responses. Biophysical Journal. 2010;98(8):1396–407.

68. Martins BM, Swain PS. Ultrasensitivity in phosphorylation-dephosphorylation cycles with little substrate. PLoS Comput Biol. 2013;9(8):e1003175.

69. Zumsande M, Gross T. Bifurcations and chaos in the mapk signaling cascade. Journal of Theoretical Biology. 2010;265(3):481–91.

70. Chickarmane V, Kholodenko BN, Sauro HM. Oscillatory dynamics arising from competitive inhibition and multisite phosphorylation. Journal of Theoretical Biology. 2007;244(1):68–76.

71. Jolley CC, Ode KL, Ueda HR. A design principle for a posttranslational biochemical oscillator. Cell Reports. 2012;2(4):938–50.

72. Feng S, Ollivier JF, Soyer OS. Enzyme sequestration as a tuning point in controlling response dynamics of signalling networks. PLoS Computational Biology. 2016;12(5):e1004918.

73. Feng S, Soyer OS. In silico evolution of signaling networks using rule-based models: Bistable response dynamics. Methods Mol Biol. 2019;1945:315–39.

74. Sarma U, Ghosh I. Different designs of kinase-phosphatase interactions and phosphatase sequestration shapes the robustness and signal flow in the mapk cascade. BMC Systems Biology. 2012;6(1):1–20.

75. Suwanmajo T, Krishnan J. Mixed mechanisms of multi-site phosphorylation. Journal of The Royal Society Interface. 2015;12(107):20141405.

76. Meinke MH, Bishop JS, Edstrom RD. Zero-order ultrasensitivity in the regulation of glycogen phosphorylase. Proceedings of the National Academy of Sciences. 1986;83(9):2865–8.

77. LaPorte DC, Koshland DE. Phosphorylation of isocitrate dehydrogenase as a demonstration of enhanced sensitivity in covalent regulation. Nature. 1983;305(5932):286–90.

78. Legewie S, Schoeberl B, Blüthgen N, Herzel H. Competing docking interactions can bring about bistability in the mapk cascade. Biophysical Journal. 2007;93(7):2279–88.

79. Dondi E, Pattyn E, Lutfalla G, Van Ostade X, Uzé G, Pellegrini S, Tavernier J. Down-modulation of type 1 interferon responses by receptor cross-competition for a shared jak kinase. Journal of Biological Chemistry. 2001;276(50):47004–12.

80. Spencer SL, Albeck JG, Burke JM, Sorger PK, Gaudet S, Kim K, Kim DH. Systemic calibration of a cell signaling network model. BMC Bioinformatics. 2010;11(1):1–14.

81. Shi T, Niepel M, McDermott JE, Gao Y, Nicora CD, Chrisler WB, Markillie LM, Petyuk VA, Smith RD, Rodland KD, et al. Conservation of protein abundance patterns reveals the regulatory architecture of the egfr-mapk pathway. Science Signaling. 2016;9(436):rs6–rs6.

82. Joslin EJ, Shankaran H, Opresko LK, Bollinger N, Lauffenburger DA, Wiley HS. Structure of the egf receptor transactivation circuit integrates multiple signals with cell context. Molecular BioSystems. 2010;6(7):1293–306.

83. Clune J, Mouret JB, Lipson H. The evolutionary origins of modularity. Proceedings of the Royal Society b: Biological Sciences. 2013;280(1755):20122863.

84. Atay O, Skotheim JM. Modularity and predictability in cell signaling and decision making. Molecular Biology of the Cell. 2014;25(22):3445–50.