

# DYNAMICS OF POST-TRANSLATIONAL MODIFICATION SYSTEMS: RECENT PROGRESS AND FUTURE DIRECTIONS

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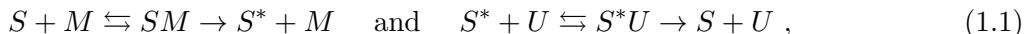
**ABSTRACT.** Post-translational modification (PTM) of proteins is important for signal transduction, and hence significant effort has gone toward understanding how PTM networks process information. This involves, on the theory side, analyzing the dynamical systems arising from such networks. Which networks are, for instance, bistable? Which networks admit sustained oscillations? Which parameter values enable such behaviors? In this Perspective, we highlight recent progress in this area and point out some important future directions. Along the way, we summarize several techniques for analyzing general networks, such as eliminating variables to obtain steady-state parametrizations, and harnessing results on how incorporating intermediates affects dynamics.

## 1. INTRODUCTION

Post-translational modification (PTM) of proteins (for instance, by phosphorylation or methylation) plays a key role in signal transduction, with disruptions having implications for human health [14, 2]. Much research therefore has focused on determining how networks of PTM's process and encode information [61, 66]. The goal, on the theory side, is to understand the dynamical systems arising from such networks. Indeed, qualitative properties, such as the capacity for bistability or oscillations, may indicate that the underlying biochemical mechanism acts as a biological switch or clock [69]. Bistability [6, 9] and oscillations [41, 43], have been observed in cellular signaling networks, such as mitogen-activated protein kinase (MAPK) cascades, in which PTM networks play a key role.

Although PTM networks are often large, with parameter values that are difficult to estimate, a growing body of research has achieved success in determining whether a given network admits, for instance, bistability or oscillations, and even, in some cases, obtaining a mathematical description of the parameter regions that give rise to such properties. The aim of this Perspective is to highlight these recent mathematical developments.

The basic building block of a PTM network is formed by the following two sets of reactions:



in which a substrate  $S$  forms a complex with a modifier  $M$ , and then  $M$  and the modified substrate  $S^*$  dissociate (left); this modification can be undone by another modifier  $U$  (right). A PTM network is adapted from Eq. 1.1 by adding known biological interactions: for instance, the complexes  $SM$  and  $S^*U$  might undergo more modifications, there might be many substrates and modifiers, and a (modified) substrate of one reaction might be a modifier of another.

Arguably the most detailed and accurate description of the dynamics of a PTM network is achieved at the level of mass-action. Here we focus on systems in which the spatial distribution of concentrations can be neglected and where models therefore take the form of ordinary differential equations. Most models in the literature are of this type. (When spatial effects may not be neglected [48, 49, 50], partial differential equations must be considered and the connection between network

structure and qualitative properties has not yet been studied to the same extent; for some recent results, see [54, 55, 65].)

Even if spatial effects are neglected, the resulting dynamical models (systems of ODEs) contain many variables and parameters and are therefore difficult to analyze. Biophysicists and biochemists have long developed tools to deal with this situation, in particular the King-Altman method and the Michaelis-Menten reduction under the quasi-steady-state assumption proposed by Briggs and Haldane (see [20, Chapters 2&4] and the references therein). These methods guide the systematic elimination of variables and thus reduce the dimension of a system. It is important to note that the steady states of these reduced systems are in one-to-one correspondence with those of the original system [29], while this need not be the case for the time-dependent trajectories of the respective ODE systems.

In recent years, the abundance of PTM networks in biology and the accompanying need to analyze large networks has stimulated further research on the systematic elimination of variables. Much of this research originated at the interface of Chemical Reaction Network Theory, mathematical biology, and algebraic geometry. Some of the results obtained thereby are a natural extension of the King-Altman method and to some extent the Michaelis-Menten reduction. In Section 3, we will summarize the most consequential such elimination results for PTM networks (after introducing the mathematical formalism used to study PTM networks in Section 2).

In Section 4 we will turn to a specific class of PTM networks, those describing the multisite phosphorylation/dephosphorylation of a single substrate. Much has been discovered in recent years about the steady states and the dynamics of these systems – more than for general PTM networks. What has been revealed is a close connection between a network’s structure and its qualitative dynamics. Consider, for instance, the sequential, double phosphorylation (also called dual-site phosphorylation) of a protein in which the enzymatic mechanism is fully *processive* versus those with fully *distributive* mechanism (see Figure 1). Processive systems are globally stable – convergent to a unique steady state – while distributive systems may be bistable. These properties – and those of several related networks – are summarized later in Figure 2.

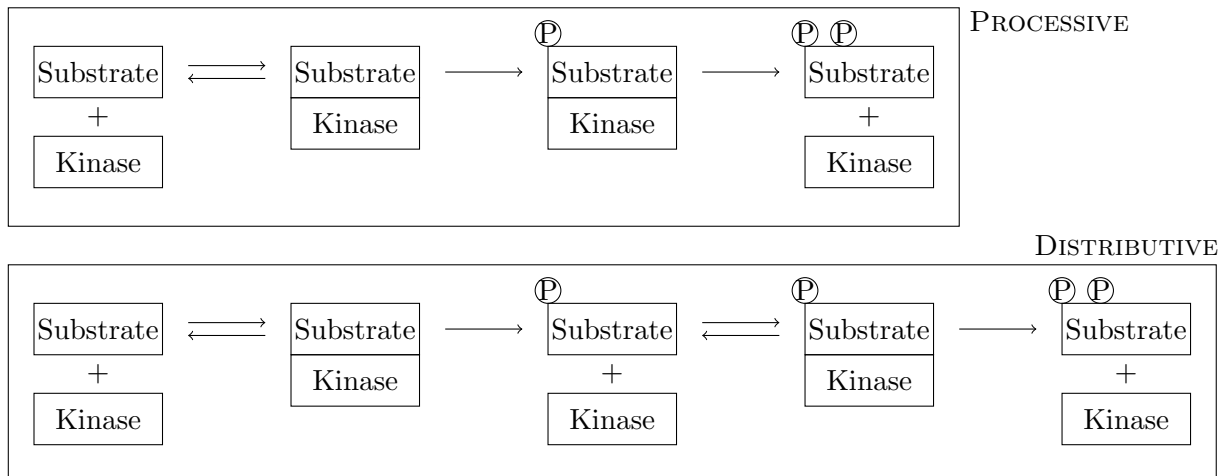


FIGURE 1. Dual-site phosphorylation may be *processive* (top) or *distributive* (bottom) or somewhere in between; cf. [64, Figure 2B].

In this Perspective, and especially at the close, we will highlight important future directions.

**1.1. Related review articles.** Excellent reviews on various aspects of PTM networks have been published (which we highlight below). Our goals therefore are complementary to what earlier reviews accomplished and, to some extent, pick up where they left off. Indeed, there is currently no review summarizing recent progress on elimination for PTM networks or the dynamics of multisite phosphorylation networks involving a single substrate.

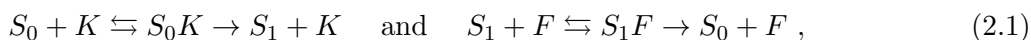
As mentioned earlier, we are interested in the question of how PTM networks process information. Theoretical aspects of this question, and experimental approaches also, were reviewed by Prabakaran *et al.* [61]. Our Perspective, in contrast, is limited to the theory side, and specifically to the question of how a PTM network constrains the resulting dynamics (which in turn constrain the capacity for information encoding).

Another related review is that of Salazar and Höfer [64], which describes the many biological functions of (multisite) phosphorylation (the best-studied PTM), a thorough list of possible mechanisms, and the resulting kinetic models. Their review, to abridge its title, goes “from molecular mechanisms to kinetic models” – whereas, here we go from kinetic models to dynamics. To be clear, Salazar and Höfer did examine dynamics (including ultrasensitivity and bistability but not oscillations), but not for all the systems described here.

Finally, much of the interest in phosphorylation networks is due to their role in MAPK cascades. The dynamics of MAPK cascades was reviewed by Hell and Rendall in [40].

## 2. BACKGROUND

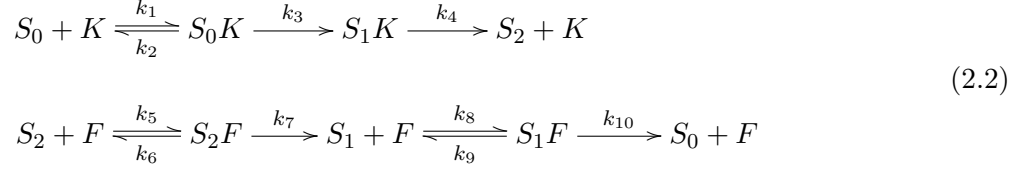
*Post-translational modification* is a biochemical alteration of a protein that occurs after its mRNA has been translated into a sequence of amino acids. The most common PTM is *phosphorylation*, the enzyme-mediated addition of a phosphate group to a protein substrate, which, to quote from [13], “can alter its behavior in almost every conceivable way.” The basic phosphorylation/dephosphorylation mechanism is a relabeled version of the building block in Eq. 1.1:



where a *kinase* and a *phosphatase* enzyme ( $K$  and  $F$ ) act as modifiers, the non-phosphorylated (unmodified) protein is denoted by  $S_0$ , and the phosphorylated (modified) protein is  $S_1$ . The mechanism in Eq. 2.1 also describes other PTMs, such as acetylation, the addition of an acetyl group. Although some PTMs are non-binary (more than one modification is possible, even at a single site [61]), we will focus on PTMs built on the mechanism in Eq. 2.1 and use, for ease, the language of phosphorylation.

So far we have discussed phosphorylation on a single site. Many substrates, however, have more than one site at which phosphate groups can be attached (in which case we continue to use subscripts to denote the number of phosphorylations, e.g.,  $S_2$  is a doubly phosphorylated protein). Such multisite phosphorylation may be *sequential* or *random*, or somewhere in between, and *processive* or *distributive*, or somewhere in between [35, 58, 64]. In *sequential* phosphorylation the last phosphate group to be attached is the first to be removed, so, for example, there is a unique once-phosphorylated substrate ( $S_1$ ). This Perspective focuses solely on sequential phosphorylation. (In *random* phosphorylation, phosphate groups can be added in any order.) *Processive* phosphorylation, as depicted in Figure 1, requires an enzyme and substrate to bind only once in order to add or remove all phosphate groups. *Distributive* phosphorylation, in contrast, requires multiple bindings to add or remove all phosphate groups, because at most one group is added with every binding of enzyme and protein.

**Example 2.1** (Mixed-mechanism network). The following is a *mixed-mechanism* dual-site network (of Figure 2(E)), in which phosphorylation is processive and dephosphorylation is distributive [66]:



Under the commonly used assumption that the (cellular) volume is constant, the network in Eq. 2.2 gives rise, via mass-action kinetics, to the following ODEs:

$$\begin{aligned}
\dot{x}_1 &= -k_1x_1x_2 + k_2x_3 + k_{10}x_9 \\
\dot{x}_2 &= -k_1x_1x_2 + k_2x_3 + k_4x_4 \\
\dot{x}_3 &= k_1x_1x_2 - (k_2 + k_3)x_3 \\
\dot{x}_4 &= k_3x_3 - k_4x_4 \\
\dot{x}_5 &= k_4x_4 - k_5x_5x_6 + k_6x_7 \\
\dot{x}_6 &= -k_5x_5x_6 - k_8x_8x_6 + (k_6 + k_7)x_7 + (k_9 + k_{10})x_9 \\
\dot{x}_7 &= k_5x_5x_6 - (k_6 + k_7)x_7 \\
\dot{x}_8 &= k_7x_7 - k_8x_6x_8 + k_9x_9 \\
\dot{x}_9 &= k_8x_6x_8 - (k_9 + k_{10})x_9 ,
\end{aligned} \tag{2.3}$$

where  $x_1, x_2, \dots, x_9$  denote, respectively, the concentrations of the species  $S_0, K, S_0K, S_1K, S_2, F, S_2F, S_1, S_1F$ . The total amounts of free and bound enzyme or substrate, denoted by  $(K_{\text{tot}}, F_{\text{tot}}, S_{\text{tot}}) \in \mathbb{R}_{>0}^3$ , remain constant as the dynamical system in Eq. 2.3 progresses, so we obtain the following *conservation laws*:

$$K_{\text{tot}} = x_2 + x_3 + x_4, \quad F_{\text{tot}} = x_6 + x_7 + x_9, \quad S_{\text{tot}} = x_1 + x_3 + x_4 + x_5 + x_7 + x_8 + x_9. \tag{2.4}$$

Thus, each trajectory  $x(t)$  of Eq. 2.3 is confined to an *invariant set* defined by some  $(K_{\text{tot}}, F_{\text{tot}}, S_{\text{tot}})$ :

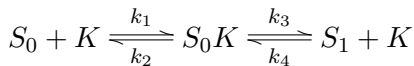
$$\{x \in \mathbb{R}_{\geq 0}^9 \mid \text{the conservation equations (2.4) hold}\}. \tag{2.5}$$

One focus of this Perspective is on the dynamics arising from multisite phosphorylation networks such as the mixed-mechanism network of Example 2.1 and those displayed in Figure 2. We report what is known about their qualitative dynamics: we describe, whether or not a network is *bistable*, i.e. whether or not it has the capacity for two or more steady states, stable to small perturbations, within some invariant set in Eq. 2.5. (The biological significance of bistability has been described, for instance, in [68]). If not, is the network *globally stable*, with a unique steady state in each invariant set to which all trajectories converge? Or, does the network admit *oscillations*, i.e., periodic orbits? To answer such questions, one key technique is elimination, which we discuss next.

3. ELIMINATION AND THE ROLE OF INTERMEDIATE COMPLEXES

We begin this section with the well-known reversible Michaelis-Menten mechanism (cf. [20]):

Reaction Network ODEs (using  $x_1$  for  $S_0$ ,  $x_2$  for  $K$ ,  $x_3$  for  $S_0K$ , and  $x_4$  for  $S_1$ ):



$$\begin{aligned} \dot{x}_1 &= -k_1x_1x_2 + k_2x_3 \\ \dot{x}_2 &= -k_1x_1x_2 + (k_2 + k_3)x_3 - k_4x_2x_4 \\ \dot{x}_3 &= k_1x_1x_2 - (k_2 + k_3)x_3 + k_4x_2x_4 \\ \dot{x}_4 &= k_3x_3 - k_4x_2x_4 \end{aligned}$$

Conservation laws

$$x_1 + x_3 + x_4 = S_{\text{tot}}$$

$$x_2 + x_3 = K_{\text{tot}}$$

The total amount of substrate  $S_{\text{tot}}$  is often much larger than that of the enzyme  $K_{\text{tot}}$  (Briggs-Haldane assumption [20]), and one can therefore assume  $\dot{x}_3 \approx 0$ . It is then standard practice to use the equation  $\dot{x}_3 = 0$  and the relation  $x_2 + x_3 = K_{\text{tot}}$  to *eliminate* the variables  $x_2$  and  $x_3$ . That is, one solves for  $x_2$  and  $x_3$  in terms of  $x_1$ ,  $x_4$ ,  $K_{\text{tot}}$ , and the rate constants (parameters):

$$x_2 = \frac{K_{\text{tot}}(k_2 + k_3)}{k_2 + k_3 + k_1x_1 + k_4x_4}, \quad x_3 = \frac{K_{\text{tot}}(k_1x_1 + k_4x_4)}{k_2 + k_3 + k_1x_1 + k_4x_4}, \quad (3.1)$$

and substitutes these expressions into  $\dot{x}_1$  and  $\dot{x}_4$  to obtain the ODEs of the reduced system:

$$\dot{x}_1 = \frac{K_{\text{tot}}(-k_1k_3x_1 + k_2k_4x_4)}{k_2 + k_3 + k_1x_1 + k_4x_4} = -\dot{x}_4.$$

Mathematically, Eq. 3.1 is a *parametrization* of  $x_2$  and  $x_3$  in terms of  $x_1$ ,  $x_4$ ,  $K_{\text{tot}}$ , and rate constants.

One can apply similar reasoning to PTM networks, removing intermediate complexes like  $S_0K$  to obtain a reduced system with fewer variables. Although this process can become arduous for large networks, the well-known King-Altman method provides a systematic way to eliminate intermediates [20]. However, this method relies at least implicitly on the ability to identify the enzyme (to guide the choice of variables to solve for). Furthermore, it does not explain the positivity of the parametrization. The first requirement, in particular, limits the applicability of the method as it is, frequently the case (for example, in cellular signaling) that the substrate of one modifier acts as a modifier for a second substrate. Such cascades cannot be handled by the King-Altman method.

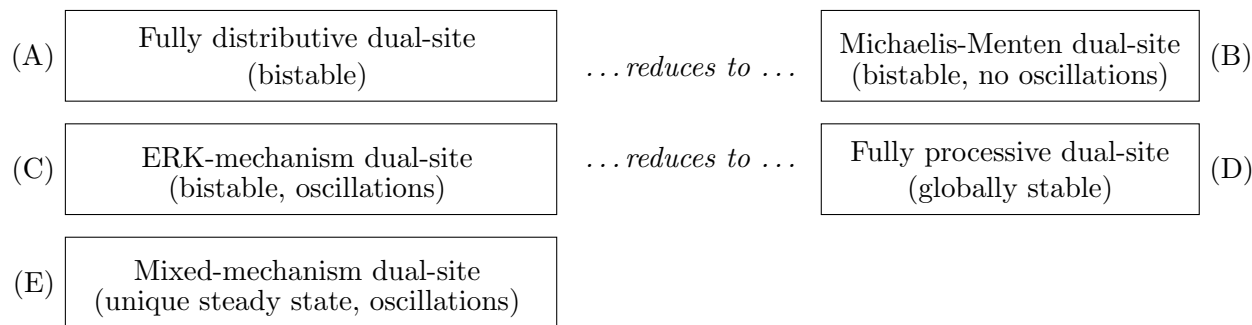


FIGURE 2. Dual-site phosphorylation/dephosphorylation networks, also called *futile cycles*: five types and their properties. A network reduces to another if the latter approximates the former if certain conditions on the parameters hold. See §4.1–§4.5.

Fortunately, both concerns were resolved recently. First, positivity in the King-Altman framework was explained by Thomson and Gunawardena [67], who initiated the theory underlying elimination in PTM networks (see also [36]). Subsequently, Feliu and Wiuf extended this to address the second concern: how to eliminate in cascades [27] and other general networks [29, 31] (see also [63]). These works describe procedures to systematically eliminate intermediates and some substrates. In fact, the resulting parametrizations arise from spanning trees of certain graphs – much like King-Altman!

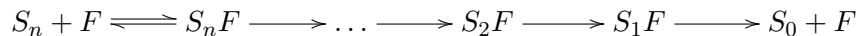
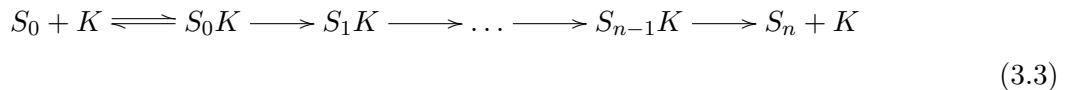
**3.1. Elimination and steady-state parametrizations.** For the Michaelis-Menten mechanism above, elimination was guided by biophysical insight as well as the aim to simplify the system and understand the dynamics (of the simplified system). If one were interested only in the positive steady states of the original ODEs, or in the number of steady states, then one might pursue a different approach which also uses a parametrization. Specifically, we set to zero the right-hand sides of the differential equations, and then solve for each  $x_i$  in terms of just  $x_2$  and  $x_4$ , and see that the set of all steady states is the 2-dimensional image of the map  $\mathbb{R}_{>0}^2 \rightarrow \mathbb{R}_{>0}^4$  given by:

$$(x_2, x_4) \mapsto \left( \frac{k_2 k_4}{k_1 k_3} x_4, x_2, \frac{k_4}{k_3} x_2 x_4, x_4 \right). \quad (3.2)$$

This *steady-state parametrization* is particularly simple, as it consists of the *monomials*  $x_2$ ,  $x_4$ , and  $x_2 x_4$  (or their scalar multiples). It is consequently called a *monomial parametrization*.

The existence of a positive parametrization is guaranteed for PTM networks [31, 59, 67]. However, such a parametrization need not be monomial.

As for the number of steady states, this corresponds mathematically to counting the number of times the image of the parametrization intersects some invariant set. If at least one such intersection (for some choice of rate constants) has at least two elements (i.e., at least two positive steady states in some invariant set), then we say that the network is *multistationary*. Deciding whether a network is multistationary is in general difficult [45], but this can be accomplished easily when the parametrization is monomial [56, 60]. Such an argument, for instance, was used to prove that a reversible version of the following *fully processive, n-site phosphorylation network* (for which the  $n = 2$  case is the network of Figure 2(D)) is *not* multistationary [19]:



Another approach to precluding multistationarity in this network is described below in Section 3.2.

For large (not necessarily PTM) networks it can be challenging to obtain a parametrization, as there is (currently) no procedure apart from computing spanning trees (unless the network has more structure [59]). Guided by experience, we suggest the following approach: if  $p$  is the number of conservation laws, then we expect to parametrize the steady states in terms of  $p$  variables (there is no guarantee though). Therefore, using a computer algebra system like `Mathematica` or `Maple`, solve for all but  $p$  variables, eliminating all intermediates (and some substrates) [31, 67]. If the result is not satisfactory, try another subset of variables.

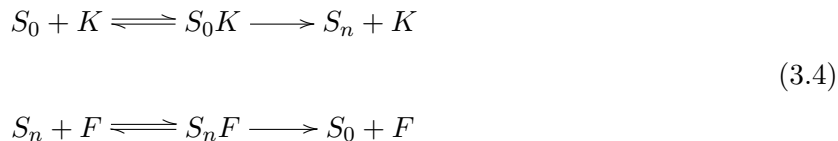
Finally, we can express a steady-state parametrization in terms of biochemically meaningful parameters, namely, the catalytic constants of the enzyme and the  $K_m$  values. For instance, for the above parametrization (3.1), there are two  $K_m$  values:  $K_{m,1} = \frac{k_2 + k_3}{k_1}$  for the substrate  $S_0$  and  $K_{m,2} = \frac{k_2 + k_3}{k_4}$  for the substrate  $S_1$ . We solve these  $K_m$  equations for  $k_1$  and  $k_4$  and substitute these

expressions into the parametrization (3.1), which becomes:

$$(x_2, x_4) \mapsto \left( \frac{k_2 K_{m,1}}{k_3 K_{m,2}} x_4, x_2, \left( 1 + \frac{k_2}{k_3} \right) \frac{1}{K_{m,2}} x_2 x_4, x_4 \right).$$

Such an approach was used to analyze how catalytic constants enable the emergence of bistability in the fully distributive dual-site phosphorylation system (the network of Figure 2(A)) [18].

**3.2. Intermediates and steady states.** Going beyond parametrizations, Feliu and Wiuf also analyzed elimination at the level of the reaction network, and its effect on steady states and their stability. Here removal of intermediates yields a reduced reaction network, and they proved the following: if the reduced network admits at least  $N \geq 1$  (locally stable) steady states (for some parameter values), then the original network also has at least  $N$  (locally stable) steady states (for some parameters) [30]. (See [8, 21, 45] for other results that “lift” multistationarity/bistability from small networks to large.) As an example, we revisit the fully processive network in Eq. 3.3. By iteratively removing intermediates  $S_i K$  and  $S_i F$  [30], we obtain the reduced network:



The network in Eq. 3.4 is mathematically equivalent to the single-site phosphorylation network (the  $n = 1$  case of the network in Eq. 3.3), which is known to be globally stable: each invariant set has a unique steady state, this steady state is positive, and it is the global attractor of the invariant set [4]. Thus, as explained above, we conclude that the network in Eq. 3.3 has at least one (locally stable) steady state.

In fact, we can say more about the network in Eq. 3.3: because it reduces to the globally stable network in Eq. 3.4, it too is globally stable [19, 24]. We proved this via results on how the dynamics are affected when intermediates are incorporated into a network. We turn to this topic next.

**3.3. Intermediates and dynamics.** As mentioned above, scientists have long used approximations of reaction systems (e.g., Michaelis-Menten), in which steady states correspond exactly to those of the original system – but trajectories need not have such a correspondence. In the case of a reaction system with intermediates, however, the trajectories are close to each other: Cappelletti and Wiuf proved that the trajectories can be uniformly approximated on compact time intervals by the trajectories of the reduced reaction system obtained by removing intermediates [11].

Similarly, global stability of a network obtained by removing intermediates, or by making some reactions irreversible, can imply that the original system also is globally stable. Marcondes de Freitas *et al.* proved such a result, using monotone systems theory [3], for removing intermediates [22, 23]; and Ali Al-Radwahi and Angeli proved such a result for making reactions irreversible [1].

**3.4. Future direction: intermediates and oscillations.** One focus of this section was on the question of how dynamics and steady states are affected when intermediates and/or reactions are added or removed. What we presently can say about dynamics, however, is mostly limited to the situation of global stability. We therefore are interested in other dynamics, such as oscillations.

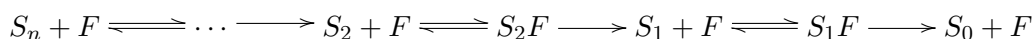
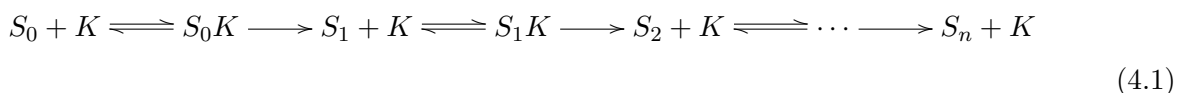
For instance, is the presence of a Hopf bifurcation preserved when reactions or intermediates are added or removed? To pose a concrete question, we revisit the mixed-mechanism network in Eq. 2.2. Suwanmajo and Krishnan showed that it admits a Hopf bifurcation; *is this still true if any of the irreversible reactions is made reversible?* Can the answer be explained by some general, yet-to-be-proven theorem that “lifts” Hopf bifurcations when reactions or intermediates are added?

Some partial results in this direction were given recently by Banaji. Regarding the question posed above, Banaji proved that making any irreversible reactions reversible indeed preserves oscillations – as do several other operations that add species or reactions [7].

#### 4. DYNAMICS OF MULTISITE PHOSPHORYLATION OF A SINGLE SUBSTRATE

This section highlights what is known – and what is open – about the dynamics of the dual-site phosphorylation systems in Figure 2. We are not attempting to be thorough – there are many more multisite systems, and we point the reader to [12, 28, 37, 44, 47, 66].

**4.1. Distributive  $n$ -site systems, including Figure 2(A).** Most studies on the mathematics of multisite phosphorylation have focused on the case of a sequential and fully *distributive* mechanism [18, 28, 42, 51, 52, 53, 57, 68, 70, 60]:



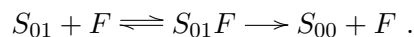
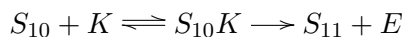
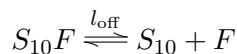
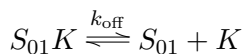
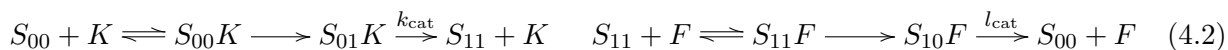
This network is multistationary for  $n \geq 2$ ; this result can be proven via a monomial parametrization of the steady states [42, 60]. As for the maximum number of positive steady states, this number is at most  $2n - 1$ ; this was proven by Wang and Sontag using an elimination similar to those in Section 3 [70]. A rationale to obtain rate constants for which the system has the maximum number of positive steady states was described in [32]. As for multiple *stable* steady states, the distributive *dual-site* network (the  $n = 2$  case of the network in Eq. 4.1) admits bistability [39].

**4.2. Distributive systems, Michaelis-Menten approximation: Figure 2(B).** The Michaelis-Menten (MM) approximation of the distributive dual-site network is a two-dimensional ODE system [71]. The validity of this approximation for phosphorylation systems has been called into question [64]. Nevertheless, analyzing this system is valuable, as dynamical properties of the MM approximation sometimes can be “lifted” to the fully distributive system [71]. Indeed, the MM approximation is bistable, and Hell and Rendall “lifted” bistability to the full system [39].

Oscillations, however, are precluded in the MM approximation, because every trajectory converges to some steady state. This was proven by Wang and Sontag [71] via monotone systems theory, and then re-proven by Bozeman and Morales [10] via the simpler Bendixson’s Criterion.

**4.3. Processive systems, including Figure 2(D).** As discussed in §3, multisite phosphorylation systems that are fully *processive* are globally stable [19, 24], and thus, in contrast to distributive systems, do *not* admit bistability or oscillations. This result revealed that processive systems lack the more interesting information-processing capabilities of distributive systems – an idea that existed in the experimental literature, but, until recently, lacked a proof.

**4.4. The ERK-mechanism system: Figure 2(C).** Figure 2(C) refers to the network of ERK regulation by dual-site phosphorylation by MEK and dephosphorylation by MKP3, here:





Here  $S_{ij}$  represents the substrate with  $i$  phosphate groups attached at the first site and  $j$  at the second. This notation reflects the fact that this network is *non-sequential*: the first site to be phosphorylated (via  $S_{00}K \rightarrow S_{01}K$ ) is also the first to be dephosphorylated ( $S_{11}F \rightarrow S_{10}F$ ).

The ERK network admits bistability and oscillations [62]. However, when  $k_{\text{cat}} \gg k_{\text{off}}$  and  $l_{\text{cat}} \gg l_{\text{off}}$ , then the network reduces to a network *without* bistability or oscillations, namely, the fully processive network in Figure 2(D). (That network comprises only the reactions in Eq. 4.2 above.) Accordingly, Rubinstein *et al.* posed the question, *How do bistability and oscillations in the ERK network emerge from the processive limit?* More precisely, when the “processivity levels”  $\frac{k_{\text{cat}}}{k_{\text{cat}}+k_{\text{off}}}$  and  $\frac{l_{\text{cat}}}{l_{\text{cat}}+l_{\text{off}}}$  are arbitrarily close to 1, is the ERK network still bistable and oscillatory?

**4.5. Mixed-mechanism systems: Figure 2(E).** Only recently have there been studies of mixed mechanisms (partially processive, partially distributive) [5]. One such network, in which phosphorylation is processive and dephosphorylation is distributive, was depicted earlier in Eq. 2.2 (Example 2.1). This network is *not* multistationary (Suwanmajo and Krishnan proved this via an elimination procedure like those described in Section 3) [66]. It follows, by a standard application of the Brouwer fixed-point theorem, that there is always a unique steady state (for all rate constants and conservation-law values). This proves half of a conjecture that we posed [19].

We were surprised when the other half of our conjecture was disproven: in contrast with processive systems (§4.3), mixed systems need *not* be globally stable. In fact, the network in Eq. 2.2 exhibits oscillations [66]! The significance of this result, as explained by its discoverers, Suwanmajo and Krishnan, is that the network in Eq. 2.2 “could be the simplest enzymatic modification scheme that can intrinsically exhibit oscillation” [66, §3.1]. Accordingly, this network’s capacity for oscillations is an interesting future direction.

As a starting point, we display in Figure 3 such oscillations arising from the mixed-mechanism network. The rate constants we used, from [66], are displayed in Table 1. Suwanmajo and Krishnan displayed oscillations for  $(K_{\text{tot}}, F_{\text{tot}}) = (17.5, 5)$ , while those in Figure 3 used  $(K_{\text{tot}}, F_{\text{tot}}) = (100, 8)$ . These results suggest that oscillations exist over a wide range of  $K_{\text{tot}}$  – and (possibly)  $F_{\text{tot}}$  – values, extending what was found earlier when only  $K_{\text{tot}}$  was allowed to vary; [66].

$k_1$	$k_2$	$k_3$	$k_4$	$k_5$	$k_6$	$k_7$	$k_8$	$k_9$	$k_{10}$
1	1	1	1	100	1	0.9	3	1	100

TABLE 1. Rate constants, from [66, Supplementary Information], leading to oscillations in the network in Eq. 2.2.

## 5. FUTURE DIRECTIONS

To add to the future directions already mentioned, here we highlight two more goals: (1) characterizing bistability (so, going beyond multistationarity) and the corresponding parameter values, and (2) developing criteria for Hopf bifurcations that are well suited to PTM networks.

Many researchers, ourselves included, have devoted much energy to analyzing multistationarity in PTM networks (and more generally in networks involving enzymes, substrates, and intermediates [59]) and others [45], but what we really are interested in here, in terms of biology, is bistability. Indeed, if an experimentalist discovered a new PTM network and wanted to know what dynamical properties it has, we have several techniques for assessing whether this network is multistationary, but embarrassingly few for determining whether it is bistable [46]. We would also want to go further, to find witnesses for bistability (values of rate constants, conservation-law values, and species concentrations at which the system is bistable) and descriptions of the parameter-space regions for which the system is bistable (or even just multistationary). Progress in this direction

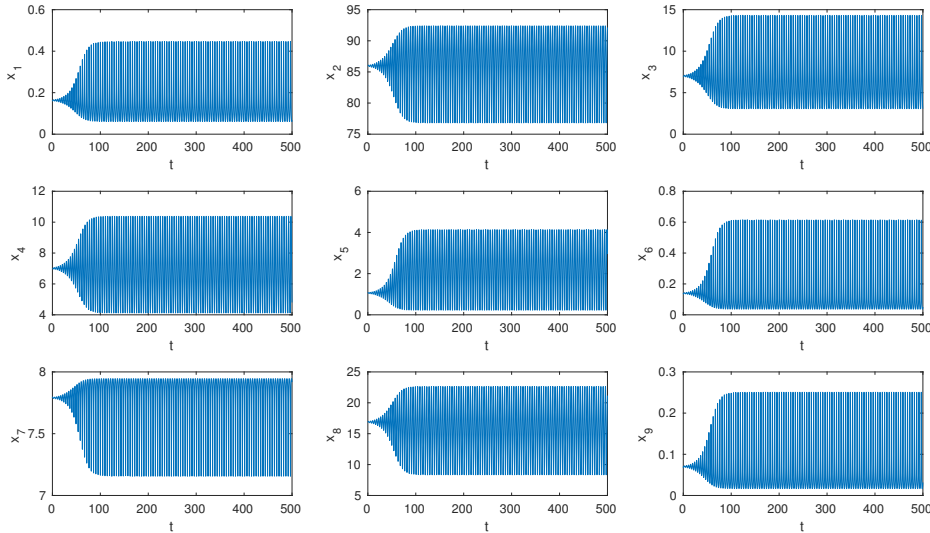


FIGURE 3. Oscillations in the mixed-mechanism network (Eq. 2.2), where rate constants are from Table 1 [66], and, in contrast with [66],  $(K_{\text{tot}}, F_{\text{tot}}, S_{\text{tot}}) = (100, 8, 40)$  and the initial values are  $x_1 = 0.16309$ ,  $x_2 = 85.978$ ,  $x_3 = 7.0112$ ,  $x_4 = 7.0112$ ,  $x_5 = 1.1$ ,  $x_6 = 0.13971$ ,  $x_7 = 7.7902$ ,  $x_8 = 16.854$ ,  $x_9 = 0.070112$ .

has been made for distributive dual-site and other PTM networks [17, 16]. Such developments have been aided by techniques from areas of mathematics such as matroid theory [42, 59], degree theory [18, 15] and numerical algebraic geometry [33, 34, 38]. We expect these and other areas to contribute in the future.

Turning our attention to oscillations, we need more tools for establishing that a network admits sustained oscillations or for precluding them. Returning to a question posed at the beginning of this Perspective, which PTM networks admit oscillations or at least a Hopf bifurcation? This area has seen some recent progress [25, 44, 62, 66], but deserves more attention.

## 6. CONCLUSIONS

What we hoped to convey in this Perspective is that the structure of a PTM network constrains its dynamics (Figure 2). This is an old idea. The question of how a (general) network constrains its dynamics was the main focus of the mathematical study of reaction systems (including chemical reaction network theory [26]) in the years prior to the scope of this Perspective. In other words, the goal was to obtain results without knowing the rate constants.

An updated version of this goal, given our interest in networks coming from biology, is to obtain results when not only are the rate constants unknown, but also aspects of the network structure, such as the precise enzymatic mechanism (Figure 1). Progress toward this goal has been aided by the methods highlighted in this Perspective – including using steady-state parametrizations and results that clarify the effect of intermediates or extra reactions. These techniques form the beginning of a theory of reaction systems well suited to any PTM or other biological signaling networks – those we already know and also those yet to be discovered.

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## REFERENCES

- [1] Muhammad Ali Al-Radhawi and David Angeli. New approach to the stability of chemical reaction networks: Piecewise linear in rates Lyapunov functions. *IEEE T. Automat. Contr.*, 61(1):76–89, 2016.
- [2] Corina Anastasaki, Anne L. Estep, Richard Marais, Katherine A. Rauen, and E. Elizabeth Patton. Kinase-activating and kinase-impaired cardio-facio-cutaneous syndrome alleles have activity during zebrafish development and are sensitive to small molecule inhibitors. *Hum. Mol. Genet.*, 18(14):2543–2554, 2009.
- [3] David Angeli, Patrick De Leenheer, and Eduardo Sontag. Graph-theoretic characterizations of monotonicity of chemical networks in reaction coordinates. *J. Math. Biol.*, 61(4):581–616, 2010.
- [4] David Angeli and Eduardo D. Sontag. Translation-invariant monotone systems, and a global convergence result for enzymatic futile cycles. *Nonlinear Anal. Real World Appl.*, 9(1):128–140, 2008.
- [5] Kazuhiro Aoki, Masashi Yamada, Katsuyuki Kunida, Shuhei Yasuda, and Michiyuki Matsuda. Processive phosphorylation of ERK MAP kinase in mammalian cells. *P. Natl. Acad. Sci. USA*, 108(31):12675–12680, 2011.
- [6] Christoph P Bagowski and James E Ferrell. Bistability in the JNK cascade. *Curr. Biol.*, 11(15):1176–1182, 2001.
- [7] Murad Banaji. Inheritance of oscillation in chemical reaction networks. *Preprint*, arXiv:1706.00684, 2017.
- [8] Murad Banaji and Casian Pantea. The inheritance of nondegenerate multistationarity in chemical reaction networks. *Preprint*, arXiv:1608.08400., 2016.
- [9] Upinder S Bhalla, Prahlad T Ram, and Ravi Iyengar. MAP kinase phosphatase as a locus of flexibility in a mitogen-activated protein kinase signaling network. *Science*, 297(5583):1018–1023, 2002.
- [10] Luna Bozeman and Adriana Morales. No oscillations in the Michaelis-Menten approximation of the dual futile cycle under a sequential and distributive mechanism. *SIAM Undergraduate Research Online*, 10:21–28, 2017.
- [11] Daniele Cappelletti and Carsten Wiuf. Uniform approximation of solutions by elimination of intermediate species in deterministic reaction networks. *Preprint*, arXiv:1609.01405., 2016.
- [12] Carlo Chan, Xinfeng Liu, Liming Wang, Lee Bardwell, Qing Nie, and Germán Enciso. Protein scaffolds can enhance the bistability of multisite phosphorylation systems. *PLOS Computational Biology*, 8(6):1–9, 06 2012.
- [13] Philip Cohen. The regulation of protein function by multisite phosphorylation—a 25 year update. *Trends Biochem. Sci.*, 25(12):596–601, 2000.
- [14] Philip Cohen. The role of protein phosphorylation in human health and disease. *Eur. J. Biochem.*, 268(19):5001–5010, 2001.
- [15] Carsten Conradi, Elisenda Feliu, Maya Mincheva, and Carsten Wiuf. Identifying parameter regions for multistationarity. *Preprint*, arXiv:1608.03993, 2016.
- [16] Carsten Conradi and Dietrich Flockerzi. Multistationarity in mass action networks with applications to ERK activation. *J. Math. Biol.*, 65(1):107–156, 2012.
- [17] Carsten Conradi, Dietrich Flockerzi, and Jörg Raisch. Multistationarity in the activation of an MAPK: parametrizing the relevant region in parameter space. *Math. Biosci.*, 211(1):105–131, 2008.
- [18] Carsten Conradi and Maya Mincheva. Catalytic constants enable the emergence of bistability in dual phosphorylation. *J. R. Soc. Interface*, 11(95), 2014.
- [19] Carsten Conradi and Anne Shiu. A global convergence result for processive multisite phosphorylation systems. *B. Math. Biol.*, 77(1):126–155, 2015.
- [20] Athel Cornish-Bowden. *Fundamentals of enzyme kinetics*. Wiley-VCH Weinheim, 2012.
- [21] Gheorghe Craciun and Martin Feinberg. Multiple equilibria in complex chemical reaction networks: extensions to entrapped species models. *IEE Proceedings-Systems Biology*, 153:179–186, 2006.
- [22] Michael Marcondes de Freitas, Elisenda Feliu, and Carsten Wiuf. Intermediates, catalysts, persistence, and boundary steady states. *J. Math. Biol.*, 74(4):887–932, 2017.
- [23] Michael Marcondes de Freitas, Carsten Wiuf, and Elisenda Feliu. Intermediates and generic convergence to equilibria. *B. Math. Biol.*, 79(7):1662–1686, 2017.
- [24] Mitchell Eithun and Anne Shiu. An all-encompassing global convergence result for processive multisite phosphorylation systems. *Math. Biosci.*, 291:1–9, 2017.
- [25] Hassan Errami, Markus Eiswirth, Dima Grigoriev, Werner M. Seiler, Thomas Sturm, and Andreas Weber. Detection of Hopf bifurcations in chemical reaction networks using convex coordinates. *J. Comput. Phys.*, 291:279–302, 2015.
- [26] Martin Feinberg. *Dynamics and Modelling of Reactive Systems*, chapter Chemical oscillations, multiple equilibria, and reaction network structure, pages 59–130. Academic Press, 1980.
- [27] Elisenda Feliu, Michael Knudsen, Lars N. Andersen, and Carsten Wiuf. An algebraic approach to signaling cascades with  $n$  layers. *Bull. Math. Biol.*, 74(1):45–72, 2012.
- [28] Elisenda Feliu and Carsten Wiuf. Enzyme-sharing as a cause of multi-stationarity in signalling systems. *J. R. Soc. Interface*, 9(71):1224–1232, 2012.

- [29] Elisenda Feliu and Carsten Wiuf. Variable elimination in chemical reaction networks with mass-action kinetics. *SIAM J. Appl. Math.*, 72(4):959–981, 2012.
- [30] Elisenda Feliu and Carsten Wiuf. Simplifying biochemical models with intermediate species. *J. R. Soc. Interface*, 10(87), 2013.
- [31] Elisenda Feliu and Carsten Wiuf. Variable elimination in post-translational modification reaction networks with mass-action kinetics. *J. Math. Biol.*, 66(1–2):281–310, 2013.
- [32] Dietrich Flockerzi, Katharina Holstein, and Carsten Conradi.  $n$ -site phosphorylation systems with  $2n - 1$  steady states. *Bull. Math. Biol.*, 76(8):1892–1916, 2014.
- [33] Elizabeth Gross, Brent Davis, Kenneth L. Ho, Daniel J. Bates, and Heather A. Harrington. Numerical algebraic geometry for model selection and its application to the life sciences. *J. R. Soc. Interface*, 13(123), 2016.
- [34] Elizabeth Gross, Heather A. Harrington, Zvi Rosen, and Bernd Sturmfels. Algebraic systems biology: a case study for the Wnt pathway. *Bull. Math. Biol.*, 78(1):21–51, 2016.
- [35] Jeremy Gunawardena. Multisite protein phosphorylation makes a good threshold but can be a poor switch. *PNAS*, 102(41):14617–14622, 2005.
- [36] Jeremy Gunawardena. A linear framework for time-scale separation in nonlinear biochemical systems. *PLoS ONE*, 7(5):e36321, 2012.
- [37] Heather A Harrington, Elisenda Feliu, Carsten Wiuf, and Michael PH Stumpf. Cellular compartments cause multistability and allow cells to process more information. *Biophys. J.*, 104(8):1824–1831, 2013.
- [38] Heather A Harrington, Dhagash Mehta, Helen M Byrne, and Jonathan D Hauenstein. Decomposing the parameter space of biological networks via a numerical discriminant approach. *preprint*, [arXiv:1604.02623](https://arxiv.org/abs/1604.02623), 2016.
- [39] Juliette Hell and Alan D. Rendall. A proof of bistability for the dual futile cycle. *Nonlinear Anal.-Real*, 24:175–189, 2015.
- [40] Juliette Hell and Alan D. Rendall. *Dynamical Features of the MAP Kinase Cascade*, pages 119–140. Springer International Publishing, Cham, 2017.
- [41] Zoe Hilioti, Walid Sabbagh, Saurabh Paliwal, Adriel Bergmann, Marcus D Goncalves, Lee Bardwell, and Andre Levchenko. Oscillatory phosphorylation of yeast Fus3 MAP kinase controls periodic gene expression and morphogenesis. *Curr. Biol.*, 18(21):1700–1706, 2008.
- [42] Katharina Holstein, Dietrich Flockerzi, and Carsten Conradi. Multistationarity in sequential distributed multisite phosphorylation networks. *B. Math. Biol.*, 75(11):2028–2058, 2013.
- [43] Huizhong Hu, Alexey Goltsov, James L Bown, Andrew H Sims, Simon P Langdon, David J Harrison, and Dana Faratian. Feedforward and feedback regulation of the MAPK and PI3K oscillatory circuit in breast cancer. *Cell. Signal.*, 25(1):26–32, 2013.
- [44] Craig C Jolley, Koji L Ode, and Hiroki R Ueda. A design principle for a posttranslational biochemical oscillator. *Cell Reports*, 2(4):938–950, 2012.
- [45] Badal Joshi and Anne Shiu. A survey of methods for deciding whether a reaction network is multistationary. *Math. Model. Nat. Phenom., special issue on “Chemical dynamics”*, 10(5):47–67, 2015.
- [46] Badal Joshi and Anne Shiu. Which small reaction networks are multistationary? *SIAM J. Appl. Dyn. Syst.*, 16(2):802–833, 2017.
- [47] Orsolya Kaputy, Debashis Barik, Maria Rosa Domingo Sananes, John J Tyson, and Béla Novák. Bistability by multiple phosphorylation of regulatory proteins. *Prog. Biophys. Mol. Bio.*, 100(1):47–56, 2009.
- [48] Boris N. Kholodenko. Cell-signalling dynamics in time and space. *Nature Reviews Molecular Cell Biology*, 7:165–, February 2006.
- [49] Boris N. Kholodenko, Guy C. Brown, and Jan B. Hoek. Diffusion control of protein phosphorylation in signal transduction pathways. *Biochem. J.*, 350(3):901–907, 2000.
- [50] Boris N. Kholodenko, John F. Hancock, and Walter Kolch. Signalling ballet in space and time. *Nat. Rev. Mol. Cell Bio.*, 11:414–426, June 2010.
- [51] Arjun Kumar Manrai and Jeremy Gunawardena. The geometry of multisite phosphorylation. *Biophys. J.*, 95(12):5533–5543, 2008.
- [52] Nick I. Markevich, Jan B. Hoek, and Boris N. Kholodenko. Signaling switches and bistability arising from multisite phosphorylation in protein kinase cascades. *J. Cell. Biol.*, 164:353 – 359, 2004.
- [53] Mercedes Pérez Millán and Adrián G. Turjanski. MAPK’s networks and their capacity for multistationarity due to toric steady states. *Math. Biosci.*, 262:125–137, 2015.
- [54] Maya Mincheva and Marc R. Roussel. Turing-Hopf instability in biochemical reaction networks arising from pairs of subnetworks. *Math. Biosci.*, 240(1):1 – 11, 2012.
- [55] F. Mohamed, C. Pantea, and A. Tudorascu. Chemical reaction-diffusion networks; convergence of the method of lines. *Preprint*, [arXiv:1704.01073](https://arxiv.org/abs/1704.01073), 2017.

- [56] Stefan Müller, Elisenda Feliu, Georg Regensburger, Carsten Conradi, Anne Shiu, and Alicia Dickenstein. Sign conditions for injectivity of generalized polynomial maps with applications to chemical reaction networks and real algebraic geometry. *Found. Comput. Math.*, 16(1):69–97, 2016.
- [57] Fernando Ortega, José L. Garcés, Francesc Mas, Boris N. Kholodenko, and Marta Cascante. Bistability from double phosphorylation in signal transduction. *FEBS J.*, 273(17):3915–3926, 2006.
- [58] Parag Patwardhan and W. Todd Miller. Processive phosphorylation: Mechanism and biological importance. *Cell. Signal.*, 19(11):2218–2226, 2007.
- [59] Mercedes Pérez Millán and Alicia Dickenstein. The structure of MESSI biological systems. *preprint*, [arXiv:1612.08763](https://arxiv.org/abs/1612.08763), 2016.
- [60] Mercedes Pérez Millán, Alicia Dickenstein, Anne Shiu, and Carsten Conradi. Chemical reaction systems with toric steady states. *B. Math. Biol.*, 74(5):1027–1065, 2012.
- [61] Sudhakaran Prabakaran, Guy Lippens, Hanno Steen, and Jeremy Gunawardena. Post-translational modification: nature’s escape from genetic imprisonment and the basis for dynamic information encoding. *Wires. Syst. Biol. Med.*, 4(6):565–583, 2012.
- [62] Boris Y. Rubinstein, Henry H. Mattingly, Alexander M. Berezhkovskii, and Stanislav Y. Shvartsman. Long-term dynamics of multisite phosphorylation. *Mol. Biol. Cell*, 27(14):2331–2340, 2016.
- [63] Meritxell Sáez, Carsten Wiuf, and Elisenda Feliu. Graphical reduction of reaction networks by linear elimination of species. *J. Math. Biol.*, 74(1–2):195–237, 2017.
- [64] Carlos Salazar and Thomas Höfer. Multisite protein phosphorylation – from molecular mechanisms to kinetic models. *FEBS Journal*, 276(12):3177–3198, 2009.
- [65] Jörg Stelling and Boris N. Kholodenko. Signaling cascades as cellular devices for spatial computations. *J. Math. Biol.*, 58(1-2):35–55, 2009.
- [66] Thapanar Suwanmajo and J. Krishnan. Mixed mechanisms of multi-site phosphorylation. *J. R. Soc. Interface*, 12(107), 2015.
- [67] Matthew Thomson and Jeremy Gunawardena. The rational parameterisation theorem for multisite post-translational modification systems. *J. Theoret. Biol.*, 261(4):626–636, 2009.
- [68] Matthew Thomson and Jeremy Gunawardena. Unlimited multistability in multisite phosphorylation systems. *Nature*, 460(7252):274–277, 2009.
- [69] John J Tyson, Reka Albert, Albert Goldbeter, Peter Ruoff, and Jill Sible. Biological switches and clocks. *J. R. Soc. Interface*, 5:S1–S8, 2008.
- [70] Liming Wang and Eduardo Sontag. On the number of steady states in a multiple futile cycle. *J. Math. Biol.*, 57(1):29–52, 2008.
- [71] Liming Wang and Eduardo D. Sontag. Singularly perturbed monotone systems and an application to double phosphorylation cycles. *J. Nonlinear Science*, 18(5):527–550, 2008.

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