LONG-TERM DYNAMICS OF MULTISITE PHOSPHORYLATION

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Abstract

Multisite phosphorylation cycles are ubiquitous in cell regulation systems and are studied at multiple levels of complexity, from molecules to organisms, with the ultimate goal of establishing predictive understanding of the effects of genetic and pharmacological perturbations of protein phosphorylation in vivo. Achieving this goal is essentially impossible without mathematical models, which provide a systematic framework for exploring dynamic interactions of multiple network components. Most of the models studied to date do not discriminate between the distinct partially-phosphorylated forms and focus on two limiting reaction regimes, distributive and processive, which differ in the number of enzyme-substrate binding events needed for complete phosphorylation or dephosphorylation. Here we use a minimal model of ERK regulation to explore the dynamics of a reaction network that includes all essential phosphorylation forms and arbitrary levels of reaction processivity. In addition to bistability, which has been studied extensively in distributive mechanisms, this network can generate periodic oscillations. Both bistability and oscillations can be realized at high levels of reaction processivity. Our work provides a general framework for systematic analysis of dynamics in multisite phosphorylation systems.
INTRODUCTION

Multisite phosphorylation cycles are ubiquitous in cell regulation systems (Lim et al., 2014). A canonical example of such a cycle is provided by the mechanism controlling the enzymatic activity of the extracellular signal-regulated kinase (ERK), an important model for studies of enzyme kinetics in cells (Shaul and Seger, 2007; Futran et al., 2013). Activation of ERK requires phosphorylation at two sites, tyrosine and threonine, within the so-called activation sequence (Payne et al., 1991; Canagarajah et al., 1997). Both sites can be phosphorylated by MEK, a dual-specificity enzyme that is essential for ERK activation in vivo (Burack and Sturgill, 1997; Ferrell and Bhatt, 1997). Both ERK and MEK exist in two isoforms in mammalian cells (ERK1/2 and MEK1/2), however the isoforms are functionally redundant (Frémin et al., 2015; Aoidi et al., 2016). ERK activation can be reversed by multiple ERK phosphatases, including PP2A (Ferrigno et al., 1993; Alessi et al., 1995), PAC1 (Ward et al., 1994; Yi et al., 1995), PTP-SL (Hendriks et al., 1995; Ogata et al., 1995; Sharma and Lombroso, 1995; Shiozuka et al., 1995; Pulido et al., 1998), HePTP (Saxena et al., 1999), MKP1 (Keyse and Emslie, 1992; Alessi et al., 1993; Sun et al., 1993; Lewis et al., 1995), MKP2 (Guan and Butch, 1995; King et al., 1995; Misra-Press et al., 1995), MKP3 (Groom et al., 1996; Mourey et al., 1996; Muda et al., 1996), and MKP4 (Dowd et al., 1998), which can either dephosphorylate one or both of the sites phosphorylated by MEK (Sohaskey and Ferrell, 1999; Zhao and Zhang, 2001; Zhou et al., 2002).

A large number of similar cycles, with varying numbers of phosphorylation sites and reaction mechanisms, were discovered by studies of intracellular networks and are actively being studied at multiple levels of complexity, from individual molecules and single reactions to organisms (Caunt et al., 2008; Salazar and Höfer, 2009; Kim et al., 2011; Prabakaran et al., 2011; Humphreys et al., 2013). The ultimate goal of these studies is to establish an integrative view of biochemical reactions in vivo, needed for predicting the effects of genetic and pharmacological perturbations of protein phosphorylation networks. For instance, activating mutations in MEK can lead to both developmental abnormalities and cancers, and drugs directly inhibiting MEK are being used in the clinic (Anastasaki et al., 2009; Caunt et al., 2015; Jindal et al., 2015). Understanding the organism-level effects of such mutations and drugs requires systematic analysis of the functional properties of multisite phosphorylation cycles and is essentially impossible without mathematical models (Qiao et al., 2007; Salazar et al., 2010; Ferrell and Ha, 2014; Piala et al., 2014; Prabakaran et al., 2014).

Several models of phosphorylation cycles have already received considerable attention from mathematicians and computational biologists (Yang et al., 2004; Salazar and Höfer, 2006; Manrai and Gunawardena, 2008; Kapuy et al., 2009; Thomson and Gunawardena, 2009; Aoki et al., 2011; Harrington et al., 2012; Aoki et al., 2013; Conradi and Mincheva, 2014a; Conradi and Mincheva, 2014b; Hell and Rendall, 2015b; Suwanmajo and Krishnan, 2015). Most of these models do not discriminate between different partially phosphorylated forms and focus on two limiting mechanisms of multisite phosphorylation: processive and distributive (Figure 1B, C). In a processive mechanism, all of the phosphorylation (or dephosphorylation) reactions happen in one enzyme-substrate binding event (Patwardhan and Miller, 2007). In a distributive mechanism, the catalytic step is followed by rapid dissociation of a partially phosphorylated substrate and subsequent reactions require a new binding event. Interestingly, multisite phosphorylation cycles formed by these two types of mechanisms appear
to have qualitatively different systems-level properties. In particular, when the mechanism is processive the network has a unique steady state for all values of model parameters (Conradi and Shiu, 2015). In contrast, models of cycles formed by distributive mechanisms admit multistability, a regime in which multiple stable steady states, with different relative amounts of the substrate phosphoforms, coexist for the same set of parameters (Wang and Sontag, 2008; Thomson and Gunawardena, 2009; Hell and Rendall, 2015a). A handful of studies have explored the systems-level properties of networks containing both limiting reaction mechanisms as reaction channels (Verdugo et al., 2013; Suwanmajo and Krishnan, 2015).

Much less is known about the general properties of models that both allow the network to operate in the regime between the processive and distributive limits (Figure 1A) and explicitly account for the existence and differential reactivities of different partially phosphorylated forms. Here we use a combination of computational approaches to explore the long-term dynamics in such a model that is motivated by structural and kinetic studies of ERK regulation. Our approach is readily applicable to a broad class of multisite phosphorylation networks.

RESULTS

Our analysis is based on a mathematical model that is motivated by biochemical studies of ERK regulation. The model describes a perfectly-mixed reaction system consisting of ERK, MEK, and MKP3, a dual specificity phosphatase that dephosphorylates both the tyrosine (Y) and threonine (T) sites within the activation sequence of ERK. Previous studies established that both MEK and MKP3 follow an ordered mechanism (Haystead et al., 1992; Zhao and Zhang, 2001). Specifically, tyrosine is the first site phosphorylated by MEK and phosphotyrosine (pY) is the first site dephosphorylated by MKP3. As a consequence of this strict order, the unphosphorylated and biphosphorylated ERK molecules (denoted by TY and pTpY, respectively) give rise to two distinct monophosphorylated forms: the first phosphorylation of ERK leads to tyrosine-phosphorylated ERK (TpY) and the first dephosphorylation of biphosphorylated ERK leads to threonine-phosphorylated ERK (pTY). At the same time, both of the monophosphorylated forms of ERK can act as substrates for both enzymes (Figure 2A).

The minimal network accounting for these interactions is closely related to the networks considered in two earlier studies. Specifically, Kholodenko and colleagues considered a network with one extra reaction, which resulted from assuming that ERK phosphorylation by MEK follows a random mechanism. Their analysis demonstrated that this network can be bistable (Markevich et al., 2004). As a consequence of this bistability, continuous variations in the relative levels of kinase and phosphatase can trigger a sharp and irreversible transition between fully unphosphorylated and biphosphorylated ERK states. Almost a decade later, as a part of computational analysis of circadian rhythms (unrelated to ERK regulation), Jolley and colleagues analyzed dynamics in a network with one more reaction, corresponding to a random dephosphorylation mechanism (Jolley et al., 2012). Based on extensive sampling of model parameters, these authors established that their network can generate self-sustained oscillations, a dynamic regime where the relative levels of different phosphorylated forms change periodically in time. Thus, the two studies most closely related to the network in Figure 2 considered
models with one or two extra reactions. As shown below, our results establish that bistability and oscillations can be found in a simpler model.

Importantly, both of these models assumed that the kinase and phosphatase follow a fully distributive mechanism, in which all catalytic steps on the way to the fully modified substrate (phosphorylated or dephosphorylated) generate dissociated enzyme and product. As a consequence, all subsequent reactions require de novo formation of complexes between the enzyme and partially modified substrates. This model can be viewed as a limiting regime of a more detailed mechanism, in which the enzyme-substrate complex is first transformed into a complex between the enzyme and product of the first reaction (Figure 1A). This complex can either dissociate or continue directly to the next catalytic step. Depending on the relative rates of these steps, this mechanism can behave as fully distributive or fully processive. When the dissociation rate constant of the newly-formed complex between the monophosphorylated substrate and enzyme is much larger than the catalytic rate constant for the subsequent reaction, the mechanism behaves as fully distributive. In our model, this corresponds to the limit in which \( k_{ub2} \gg k_2 \), which makes the kinase fully distributive, and \( k_{ub4} \gg k_4 \), which makes the phosphatase fully distributive (see Table I for the assignment of rate constants to reaction steps). In the opposite extreme, when the catalytic reaction is much faster than dissociation, the mechanism behaves as processive (\( k_{ub2} \ll k_2 \) and \( k_{ub4} \ll k_4 \)). Between these extremes, the mechanism, which can be called “mixed,” can accommodate both distributive and processive reaction channels.

We used the mixed reaction mechanism as a building block in constructing the minimal model of ERK regulation (Figure 2B). This model describes six enzymatic reactions that interconvert four different forms of the substrate. To simplify the notation, in the rest of the presentation these states are denoted by \( S_{00}, S_{01}, S_{10}, S_{11} \), which correspond to the unphosphorylated (TY), two monophosphorylated (TpY and pTY), and bisphosphorylated (pTpY) molecules. The two enzymes are denoted by \( E \) and \( F \), corresponding to MEK and MKP3, respectively. The mass action model of this network leads to a system of 12 coupled ODEs describing the joint dynamics of 2 enzymes (\( E \) and \( F \)), 4 substrates (\( S_{00}, S_{01}, S_{10}, S_{11} \)), and 6 enzyme-substrate complexes (\( ES_{00}, ES_{01}, ES_{10}, FS_{01}, FS_{10}, FS_{11} \)).

The model has 21 free parameters: three rate constants for each of the six enzymatic reactions and three total concentrations of the substrate and two enzymes (\( S_{tot}, E_{tot}, \) and \( F_{tot} \)). In the limit when enzymes are much less abundant than substrates (\( E_{tot} \ll S_{tot} \) and \( F_{tot} \ll S_{tot} \)), the dynamics of the six complexes are slaved to the dynamics of the free substrates, and the model can be reduced to a system of only four ODEs for the substrates (\( S_{00}, S_{01}, S_{10}, S_{11} \)). We developed an efficient approach for applying the steady state approximation for the complexes and deriving reduced models in this and related classes of enzyme-substrate networks. As a result of the steady state approximation, the dynamics of the four remaining variables in the reduced model satisfy the conservation equation \( S_{00} + S_{01} + S_{10} + S_{11} = 1 \), after rescaling the substrate concentrations by \( S_{tot} \). Thus, the dynamics in the reduced model are effectively three-dimensional and can be readily visualized.

As a starting point for analyzing the dynamics, we used a combination of computational and algebraic techniques to characterize the steady states in the reduced model. The right hand sides of the four ODEs contain rational functions of the four variables, and the corresponding steady state problem gives rise to
a system of four coupled quadratic equations. Using Gröbner bases, a tool from algebraic geometry that has previously been applied in the context of dual phosphorylation cycles for mechanism discrimination (Gunawardena, 2007; Manrai and Gunawardena, 2008; Thomson and Gunawardena, 2009; Cox et al., 2010; Harrington et al., 2012), all solutions of this system of polynomials can be calculated for any given set of model parameters. Furthermore, the stability of the resulting steady states can be readily evaluated by examining the eigenvalues of the linearized problem. We implemented this algorithm in Mathematica (Wolfram Research, IL) and used it to examine the steady states in our model for $5 \times 10^5$ parameter vectors randomly drawn uniformly in logarithm over the 20-dimensional space of model parameters. The Supplemental Materials contain the Mathematica notebooks used to implement parameter sampling and evaluation of long-term dynamics.

Our analysis revealed three classes of outcomes (Figure 3). The most abundant class consists of parameter vectors that correspond to steady states that are unique and linearly stable (Figure 3A). The second class contains parameter vectors that predict three steady states, two stable and one unstable (Figure 3B). Of the $5 \times 10^5$ parameter sets sampled, 1,877 fell into this class, corresponding to a hit rate of roughly 1 in 270. Finally, the third and least abundant class of outcomes corresponds to parameter vectors that result in steady states that are unique and linearly unstable. In this case, the linearized problem has at least one eigenvalue with positive real part. This means that any small perturbation to the steady state solution should grow, leading to a periodic solution due to the conservation condition for the total amount of substrate. In principle, it is possible that some of such parameter sets could correspond to more complex dynamics, such as deterministic chaos, but our analysis so far has not revealed such behaviors. By time integration of the full nonlinear problem (without making the steady state approximation for complexes), we confirmed that models with parameters in this class give rise to stable limit cycles, in which the relative abundances of the four phosphoforms change periodically in time (Figure 3C). Only 120 parameter sets were found that fell into this class, corresponding to a hit rate of about 1 in 4,300. The Supplemental Materials contain Matlab (The MathWorks, Natick, MA) code for numerically integrating the equations of the full and reduced systems at the parameter values used to generate Figure 3.

The three classes of long-term dynamics identified by our computational screening of model parameters suggest that our network can display three different classes of input-output behaviors. We define the input to be the ratio of the total amounts of kinase and phosphatase ($\rho = E_{tot}/F_{tot}$) and the output to be the long-term concentration of dually-phosphorylated substrate, $S_{11}$. To illustrate this, we used numerical continuation algorithms to compute the branches of steady states and limit cycles as a function of the input to the network (Figure 4). As the starting points for numerical continuation, we used the steady states shown in Figure 3, the parameters for which are given in Table II. First, we verified that the nonlinear behaviors found in these three cases using the pseudo steady state approximation carried back to the full model. Then, the steady state branches were calculated using the full model. The three parameter sets with different types of long-term dynamics gave rise to three different types of steady state branches. In all three cases, the state of the substrate transitions from the unphosphorylated form to the fully phosphorylated form as the input to the network is increased, but this transition can pass through robust regions of bistability or oscillations.
To summarize, our analysis of the minimal mechanism of the ERK regulation cycle reveals that the system can function as an irreversible switch and as an oscillator. Both of these regimes were previously detected in cycles with additional reactions (Markevich et al., 2004; Jolley et al., 2012). Our results demonstrate that oscillations and bistability persist in a simpler model that is based on the interactions and reactions established in the MEK/ERK/MKP3 system (Haystead et al., 1992; Zhao and Zhang, 2001; Zhou et al., 2002; Aoki et al., 2013). We found that bistability and oscillations in our model can be realized at very different levels of reaction processivity, which can be defined as the probability that a complex between the partially phosphorylated substrate and enzyme will not dissociate and will continue to the next reaction step.

For instance, for the parameter set corresponding the limit cycle shown in Figure 5A, both the phosphorylation and dephosphorylation reactions are highly processive—for both of these reactions the rate constant of the second catalytic step exceeds, by orders of magnitude, the dissociation rate constant for the partially phosphorylated form. As a consequence, the relative amounts of the monophosphorylated forms at any given time are very low, and the substrate switches between the unphosphorylated and bisphosphorylated forms. A limit cycle in the strongly distributive regime, when partially phosphorylated forms are much more likely to dissociate than continue to the next catalytic step, has a very different structure (Figure 5B). Here all of the four possible phosphoforms are present at appreciable levels at different parts of the oscillating trajectory. Of the 1,877 parameter sets that produce bistability, 18 were found for which the probability of the processive reaction channel is at least as likely as the distributive one ($k_2 \geq k_{ub2}$ and $k_4 \geq k_{ub4}$). However, only one parameter set of the 120 that were found to produce oscillations satisfies this condition.

Note that earlier analysis of a model that does not distinguish between different partially phosphorylated forms conclusively ruled out bistability in the fully processive regime (Conradi and Shiu, 2015). At the same time, our results obtained for a model with multiple partially phosphorylated forms found that both bistability and oscillations can be realized at high levels of processivity. In the future, it will be interesting to determine how close to the fully processive limit can bistable or oscillatory regimes still exist.

DISCUSSION

We used an idealized mechanism of ERK regulation to explore the long-term dynamics of a model that accounts for all relevant phosphorylation forms and nonzero levels of reaction processivity. In addition to bistability, which has been studied extensively in distributive mechanisms, we found that this model can also generate oscillations. Whether such oscillations can be realized in a single ERK regulation cycle is still unclear, but similar oscillations, with ordered appearance of four distinct phosphorylation states, form the basis for robust circadian rhythms in cyanobacteria (Rust et al., 2007). At the same time, several lines of evidence suggest that ERK phosphorylation in cells can be switch-like, in the sense that most of ERK is in fully dephosphorylated or dually phosphorylated forms (Hahn et al., 2013). In order to interpret these observations from studies in cells, our model must be extended to include additional components (Harrington et al., 2013; Michailovici et al., 2014; Shindo et al., 2016) and interactions, such as the possibility of ERK dephosphorylation by multiple phosphatases (Rintelen et al., 2003).
Another important direction is to include the effects of intracellular crowding, which slows down diffusion. This may result in increased rebinding of partially phosphorylated substrate to the same enzyme. As a consequence the processive reaction channel becomes more prominent, which may in turn lead to significant changes in the domain of bistability (Takahashi et al., 2010; Gopich and Szabo, 2013; Verdugo et al., 2013; Gopich and Szabo, 2016). Our approach should be readily applicable to kinetic models that include these additional effects, as well as the effects of intracellular compartments and ERK interaction with its substrates, which can protect of ERK from phosphatases (Kim et al., 2011; Liu et al., 2011).

Note that while there are multiple techniques for probing the long-term dynamics in multisite phosphorylation cycles, there are no general-purpose tools for probing their transient responses to time-varying inputs. Nevertheless, analysis of transient responses is essential for understanding many of the functional properties of phosphorylation cycles, including their roles during inductive signaling in developing tissues (Lim et al., 2015; Mattingly et al., 2015). In the future, it will be interesting to classify transient responses of multisite phosphorylation cycles to several of the typical inputs encountered by these networks in vivo, starting with pulses provided by the upstream signaling components.

One may ask whether the nonlinear behaviors we found in the model can occur at biologically feasible parameter values. We argue that this question motivates experiments in which isolated components of phosphorylation cycles, such as the ERK-MEK-MKP3 system, are reconstituted in-vitro for the purpose of constraining model parameters and determining which long-term behaviors are feasible in such systems.

Another important direction for future work is the systematic analysis of mutations affecting the components of phosphorylation cycles. As an example, it is known that ERK uses the same docking domain to bind to the enzymes that phosphorylate and dephosphorylate it (Tanoue et al., 2000). A well-studied mutation in this domain, which should result in decreased affinity for both kinase and phosphatase, acts as gain of function in vivo (Brunner et al., 1994). Quantitative explanation for this effect is still lacking, but it is generally accepted that ERK binding to a phosphatase, such as MKP3, is affected to a greater extent than its binding to the activating enzyme (Bott et al., 1994; Zhou and Zhang, 1999; Zhao and Zhang, 2001; Zhou et al., 2002; Zhang et al., 2003). The relative effects of this mutation on ERK’s interactions with the two enzymes have not been quantified, but once this is done, our approach can be used to predict how these effects influence the systems-level properties of the dual phosphorylation cycle that controls ERK with this mutation.
METHODS

Mathematical model of ERK regulation

The following system of equations describe the dynamics of a kinase (E), a phosphatase (F), a substrate with four phosphorylation states \((S_{00}, S_{01}, S_{10}, S_{11})\), and 6 enzyme-substrate complexes \((ES_{00}, ES_{01}, ES_{10}, FS_{01}, FS_{10}, FS_{11})\). Here, each index on the substrates and complexes is a phosphorylation site, with 0 denoting unphosphorylated and 1 denoting phosphorylated.

\[
\frac{d[S_{00}]}{dt} = -k_{b1}[E][S_{00}] + k_{ub1}[ES_{00}] + k_{4}[FS_{10}] + k_{5}[FS_{01}] \tag{1.1}
\]

\[
\frac{d[S_{01}]}{dt} = -k_{b2}[E][S_{01}] + k_{ub2}[ES_{01}] - k_{b5}[F][S_{01}] + k_{ub5}[FS_{01}] \tag{1.2}
\]

\[
\frac{d[S_{10}]}{dt} = -k_{b4}[F][S_{10}] + k_{ub4}[FS_{10}] - k_{b6}[E][S_{10}] + k_{ub6}[ES_{10}] \tag{1.3}
\]

\[
\frac{d[S_{11}]}{dt} = -k_{b3}[F][S_{11}] + k_{ub3}[FS_{11}] + k_{2}[ES_{01}] + k_{6}[ES_{10}] \tag{1.4}
\]

\[
\frac{d[ES_{00}]}{dt} = k_{b1}[E][S_{00}] - (k_{ub1} + k_{1})[ES_{00}] \tag{1.5}
\]

\[
\frac{d[ES_{01}]}{dt} = k_{b2}[E][S_{01}] - (k_{ub2} + k_{2})[ES_{01}] + k_{1}[ES_{00}] \tag{1.6}
\]

\[
\frac{d[ES_{10}]}{dt} = k_{b6}[E][S_{10}] - (k_{ub6} + k_{6})[ES_{10}] \tag{1.7}
\]

\[
\frac{d[ES_{11}]}{dt} = k_{b5}[F][S_{11}] - (k_{ub5} + k_{5})[FS_{11}] \tag{1.8}
\]

\[
\frac{d[FS_{10}]}{dt} = k_{b4}[F][S_{10}] - (k_{ub4} + k_{4})[FS_{10}] + k_{3}[FS_{11}] \tag{1.9}
\]

\[
\frac{d[FS_{11}]}{dt} = k_{b3}[F][S_{11}] - (k_{ub3} + k_{3})[FS_{11}] \tag{1.10}
\]

\[
\frac{d[E]}{dt} = -k_{b1}[E][S_{00}] + k_{ub1}[ES_{00}] - k_{b2}[E][S_{01}]
\]

\[
+(k_{ub2} + k_{2})[ES_{01}] - k_{b6}[E][S_{10}] + (k_{ub6} + k_{6})[ES_{10}] \tag{1.11}
\]

\[
\frac{d[F]}{dt} = -k_{b5}[F][S_{11}] + (k_{ub5} + k_{5})[FS_{11}] - k_{b4}[F][S_{10}]
\]

\[
+(k_{ub4} + k_{4})[FS_{10}] - k_{b3}[F][S_{11}] + k_{ub3}[FS_{11}] \tag{1.12}
\]

Conservation laws:

\[
E_{tot} = [E] + [ES_{00}] + [ES_{01}] + [ES_{10}] \tag{1.13}
\]

\[
F_{tot} = [F] + [FS_{01}] + [FS_{10}] + [FS_{11}] \tag{1.14}
\]
\[ S_{\text{tot}} = [S_{00}] + [S_{01}] + [S_{10}] + [S_{11}] \]
\[ + [ES_{00}] + [ES_{01}] + [ES_{10}] + [FS_{01}] + [FS_{10}] + [FS_{11}] \]

Matrix representation

It is convenient to write the above system of equations in vector-matrix form.

\[
S = \begin{bmatrix} [S_{00}] \\ [S_{01}] \\ [S_{10}] \\ [S_{11}] \end{bmatrix}, \quad C^k = \begin{bmatrix} [ES_{00}] \\ [ES_{01}] \\ [ES_{10}] \end{bmatrix}, \quad C^p = \begin{bmatrix} [FS_{01}] \\ [FS_{10}] \\ [FS_{11}] \end{bmatrix},
\]

(2.1-2.3)

\[
K^k_{b1} = \begin{bmatrix} k_{b1} & 0 & 0 & 0 \\ 0 & k_{b2} & 0 & 0 \\ 0 & 0 & k_{b6} & 0 \\ 0 & 0 & 0 & 0 \end{bmatrix}, \quad K^p_{b1} = \begin{bmatrix} 0 & 0 & 0 & 0 \\ 0 & k_{b5} & 0 & 0 \\ 0 & 0 & k_{b4} & 0 \\ 0 & 0 & 0 & k_{b3} \end{bmatrix},
\]

(2.4, 2.5)

\[
K^k_1 = \begin{bmatrix} k_{ub1} & 0 & 0 & 0 \\ 0 & k_{ub2} & 0 & 0 \\ 0 & 0 & k_{ub6} & 0 \\ 0 & k_2 & k_6 \end{bmatrix}, \quad K^p_1 = \begin{bmatrix} k_5 & k_4 & 0 & 0 \\ k_{ub5} & 0 & 0 & 0 \\ 0 & k_{ub4} & 0 & 0 \\ 0 & 0 & 0 & k_{ub3} \end{bmatrix},
\]

(2.6, 2.7)

\[
K^k_{b2} = \begin{bmatrix} k_{b1} & 0 & 0 & 0 \\ 0 & k_{b2} & 0 & 0 \\ 0 & 0 & k_{b6} & 0 \\ 0 & 0 & 0 & 0 \end{bmatrix}, \quad K^p_{b2} = \begin{bmatrix} 0 & 0 & 0 & 0 \\ 0 & k_{b5} & 0 & 0 \\ 0 & 0 & k_{b4} & 0 \\ 0 & 0 & 0 & k_{b3} \end{bmatrix},
\]

(2.7, 2.8)

\[
K^k_2 = \begin{bmatrix} k_1 + k_{ub1} & 0 & 0 & 0 \\ -k_1 & k_2 + k_{ub2} & 0 & 0 \\ 0 & 0 & k_6 + k_{ub6} \end{bmatrix},
\]

(2.9)

\[
K^p_2 = \begin{bmatrix} k_5 + k_{ub5} & 0 & 0 & 0 \\ 0 & k_4 + k_{ub4} & -k_3 & 0 \\ 0 & 0 & k_3 + k_{ub3} \end{bmatrix}.
\]

(2.10)

In terms of these vectors and matrices, the differential equations become:

\[
\frac{dS}{dt} = -[E]K^k_{b1}S + K^k_1C^k - [F]K^p_{b1}S + K^p_1C^p,
\]

(2.11)

\[
\frac{dc^k}{dt} = [E]K^k_{b2}S - K^k_2C^k,
\]

(2.12)

\[
\frac{dc^p}{dt} = [F]K^p_{b2}S - K^p_2C^p,
\]

(2.13)

\[
\frac{d[E]}{dt} + T \left( \frac{dc^k}{dt} \right) = 0,
\]

(2.14)

\[
\frac{d[F]}{dt} + T \left( \frac{dc^p}{dt} \right) = 0.
\]

(2.15)
Here we define the operator $T(\cdot)$, which acts on a vector and takes the sum of the elements of the vector. The conservation laws become:

$$E_{tot} = [E] + T(C^k),$$
(2.16)

$$F_{tot} = [F] + T(C^p),$$
(2.17)

$$S_{tot} = T(S) + T(C^k) + T(C^p).$$
(2.18)

**Pseudo steady state approximation**

In the pseudo steady state approximation (valid for $E_{tot}, F_{tot} \ll S_{tot}$), we assume that the left hand side of the differential equations for the complexes vanish, giving:

$$C^k = [E](K^k_2)^{-1}K^k_{b2}S,$$
(3.1)

$$C^p = [F](K^p_2)^{-1}K^p_{b2}S.$$  
(3.2)

Substituting these expressions (Eqns 3.1 and 3.2) into the conservation laws for $[E]$ and $[F]$ (Eqns 2.16 and 2.17) and solving for each enzyme concentration gives:

$$[E] = \frac{E_{tot}}{1+T((K^k_2)^{-1}K^k_{b2}S)},$$
(3.3)

$$[F] = \frac{F_{tot}}{1+T((K^p_2)^{-1}K^p_{b2}S)}.$$  
(3.4)

Substituting the expressions for the enzymes and the complexes into the differential equations for the substrate concentrations gives the reduced system of differential equations:

$$\frac{dS}{dt} = \frac{E_{tot}(-K^k_{b1}+K^k_1(K^k_2)^{-1}K^k_{b2})S}{1+T((K^k_2)^{-1}K^k_{b2}S)} + \frac{F_{tot}(-K^p_{b1}+K^p_1(K^p_2)^{-1}K^p_{b2})S}{1+T((K^p_2)^{-1}K^p_{b2}S)}.$$  
(3.5)

Lumping together the matrices in the numerator and the denominator gives:

$$L^k = -K^k_{b1} + K^k_1(K^k_2)^{-1}K^k_{b2},$$
(3.6)

$$L^p = -K^p_{b1} + K^p_1(K^p_2)^{-1}K^p_{b2},$$
(3.7)

$$M^k = (K^k_2)^{-1}K^k_{b2},$$
(3.8)

$$M^p = (K^p_2)^{-1}K^p_{b2},$$
(3.9)

$$\frac{dS}{dt} = \frac{E_{tot}L^kS}{1+T(M^kS)} + \frac{F_{tot}L^pS}{1+T(M^pS)}.$$  
(3.10)
At this point we will also nondimensionalize the system. First, we rescale all substrate and complex concentrations by $S_{tot}$. However, the concentrations of the complexes are bounded above by the total concentration of enzyme, $E_{tot}$. Additionally, we are taking the limit in which $E_{tot}/S_{tot}$ goes to zero. Therefore, the complexes disappear from the conservation law for the substrate (Eqn 2.18), and the equation becomes:

$$1 = T(S).$$  \hspace{1cm} (3.11)

Rescaling time by $k_3 F_{tot}/S_{tot}$, $L^k$ and $L^p$ by $k_3/S_{tot}$, $M^k$ and $M^p$ by $S_{tot}$, and introducing the parameter $\rho = E_{tot}/F_{tot}$ gives the dimensionless system of differential equations:

$$\frac{dS}{dt} = \frac{\rho L^k S}{1 + T(M^k S)} + \frac{L^p S}{1 + T(M^p S)},$$  \hspace{1cm} (3.12)

where all quantities are now understood to be in their dimensionless form.

**Sampling of model parameters**

Parameters were sampled uniformly in logarithm from a 20-dimensional hypercube. All rate constants were allowed to take on values between $10^{-1}$ and $10^7$. The total concentrations of kinase and phosphatase were allowed to vary between $10^{-4}$ and $10^{-1}$.

**Steady state calculations**

In order to solve for the steady states of the system of differential equations shown above, we set the left hand sides equal to zero:

$$0 = \frac{\rho L^k S}{1 + T(M^k S)} + \frac{L^p S}{1 + T(M^p S)},$$  \hspace{1cm} (4.1)

which is equivalent to:

$$0 = \rho [1 + T(M^p S)] L^k S + [1 + T(M^k S)] L^p S.$$  \hspace{1cm} (4.2)

It should be noted that this is a system of algebraic polynomial equations in the substrate concentrations, with each polynomial being at most degree 2. Using the computer algebra software *Mathematica* (Wolfram Research, IL), it is possible to numerically find all solutions to this system of equations. This is done by computation of a Gröbner basis using an efficient monomial ordering, followed by eigensystem methods to extract numerical roots (Cox *et al.*, 2010). The Gröbner basis for a system of polynomials is an equivalent polynomial system that has many useful properties. For example, the set of polynomials in the Gröbner basis has the same set of roots as the original polynomials. In the simplest case, for a linear function of any number of variables, the Gröbner basis computation is equivalent to the Gaussian elimination procedure.

Among the solutions to the polynomial system of equations in (Eqn 4.2), the only ones kept were those that had no negative components in the vector $S$ and obeyed conservation of substrate (Eqn 3.11). Continuations of steady states were performed in Matcont (Dhooge *et al.*, 2003), the numerical continuation software for *Matlab* (The MathWorks, Natick, MA).
Linear stability analysis

If we define \( f(S) = \frac{dS}{dt} = \rho L^k S + \frac{L^P S}{1 + T(M^P S)} \), then determining the linear stability of a steady state \( \bar{S} \) of the reduced system of differential equations requires calculation of the eigenvalues of the Jacobian matrix \( J(\bar{S}) = \frac{df}{dS} \bar{\bar{S}} \), evaluated at that steady state. The equation for \( J(\bar{S}) \) in terms of the matrices defined in the previous section is:

\[
J(\bar{S}) = \rho \left( \frac{L^k}{1 + T(M^k \bar{S})} - \frac{(L^k \bar{S})^T (M^k)}{(1 + T(M^k \bar{S}))^2} \right) + \frac{L^P}{1 + T(M^P \bar{S})} - \frac{(L^P \bar{S})^T (M^P)}{(1 + T(M^P \bar{S}))^2}
\]

\[
= \rho J^k(\bar{S}) + J^P(\bar{S}). \tag{5.1}
\]

Here, \( T(M^k) \) (and likewise \( T(M^P) \)) means the sum of the columns of \( M^k \), producing a row vector, so that the product \( (L^k \bar{S})^T (M^k) \) is the outer product of the column vector \( (L^k \bar{S}) \) and the row vector \( T(M^k) \). The denominator of each term is a scalar.

For each parameter set, the linear stability of each nonnegative steady state solution that satisfied the conservation condition was evaluated by substituting it into Eqn 5.1 and calculating the eigenvalues of the Jacobian matrix \( J(\bar{S}) \). If one or more eigenvalues had positive real part, then the steady state was linearly unstable. If all eigenvalues had negative real parts, then the steady state was linearly stable.

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Figure 1: Mixed, processive, and distributive mechanisms. $S_{00}, S_{01},$ and $S_{11}$ denote unphosphorylated, monophosphorylated, and bisphosphorylated forms of the substrate. A) In our model, dual phosphorylation and dephosphorylation were assumed to occur by a mixed mechanism. Processive and distributive mechanisms are limiting cases of the mixed mechanism. B) In a processive mechanism, the same enzyme phosphorylates the substrate twice, without dissociating. This corresponds to the limit where $k_{cat}$ is much larger than $k_{off}$ for the complex of enzyme and monophosphorylated substrate. C) In a distributive mechanism, upon the first phosphorylation event, the enzyme-substrate complex dissociates immediately, allowing the molecules to find new binding partners. This corresponds to the limit where $k_{off}$ is much larger than $k_{cat}$ for the complex of enzyme and monophosphorylated substrate.

Figure 2: Reaction network (A) and reaction mechanism (B) in the minimal model of ERK regulation by MEK and MKP3. Rate constants associated with each reaction step in (B) are given in Table I.

Figure 3: Three types of long term dynamics in the model: a unique stable steady state (A), bistability (B), and a stable limit cycle (C). Colors are trajectories in phase space from multiple initial conditions. Black dots correspond to stable steady states and red dots to unstable steady states. The black trajectory in (C) corresponds to the stable limit cycle, which all initial conditions approach after long times. The inset in (C) zooms in near the unstable steady state, showing that the limit cycle orbit passes very close by the unstable steady state. Phase plots were constructed by numerically integrating the full model. Parameter values can be found in Table II.

Figure 4: Branches of steady states as a function of the ratio of the total amounts of kinase and phosphatase, $\rho = E_{tot}/F_{tot}$. All parameters other than the total kinase concentration are the same as those in Table II. A) The system only has a unique stable steady state for all values of $\rho$. B) The system has two stable steady states for some values of $\rho$ and exhibits switch-like behavior and hysteresis. C) The system exhibits limit cycles for a range of values of $\rho$. Close to the left bifurcation point, two stable limit cycles can coexist, separated by unstable limit cycles. The Supplement contains Matlab code for numerically integrating the equations at the parameters and total enzyme concentrations for which two stable limit cycles coexist. The inset zooms in on the region near the left bifurcation point. Note that in all three cases shown here, numerical continuation was performed on the full model of the dual phosphorylation cycle, showing that the nonlinear behaviors found under the pseudo steady state approximation lift back to the full model in most cases. Additionally, $E_{tot}$ and $F_{tot}$ are both much smaller than $S_{tot}$ for all values of $\rho$ in these bifurcation diagrams.
Figure 5: Periodic solutions were found at two different levels of reaction processivity. Time courses and state space plots of limit cycles found in the processive (A,) and distributive (B) regimes. In both cases, both the kinase and phosphatase branches operate in the regime where the processive reaction channel is at least as probable as the distributive one. Time courses were generated by numerically integrating the full model. Parameter values can be found in Table II.
Table I

| Rate Constant | Reaction                  |
|---------------|---------------------------|
| $k_1$         | $ES_{00} \rightarrow ES_{01}$ |
| $k_2$         | $ES_{01} \rightarrow E + S_{11}$ |
| $k_3$         | $FS_{11} \rightarrow FS_{10}$ |
| $k_4$         | $FS_{10} \rightarrow F + S_{00}$ |
| $k_5$         | $FS_{01} \rightarrow F + S_{00}$ |
| $k_6$         | $ES_{10} \rightarrow E + S_{11}$ |
| $k_{b1}$      | $E + S_{00} \rightarrow ES_{00}$ |
| $k_{b2}$      | $E + S_{01} \rightarrow ES_{01}$ |
| $k_{b3}$      | $F + S_{11} \rightarrow FS_{11}$ |
| $k_{b4}$      | $F + S_{10} \rightarrow FS_{10}$ |
| $k_{b5}$      | $F + S_{01} \rightarrow FS_{01}$ |
| $k_{b6}$      | $E + S_{10} \rightarrow ES_{10}$ |
| $k_{ub1}$     | $ES_{00} \rightarrow E + S_{00}$ |
| $k_{ub2}$     | $ES_{01} \rightarrow E + S_{01}$ |
| $k_{ub3}$     | $FS_{11} \rightarrow F + S_{11}$ |
| $k_{ub4}$     | $FS_{10} \rightarrow F + S_{10}$ |
| $k_{ub5}$     | $FS_{01} \rightarrow F + S_{01}$ |
| $k_{ub6}$     | $ES_{10} \rightarrow E + S_{10}$ |

Rate constants associated with each reaction step in the reaction diagram in Figure 2B.
### Table II

| Parameter | Fig 3A, 4A – Monostable | Fig 3B, 4B – Bistable | Fig 3C, 4C, Fig 5A – Limit cycle | Fig 5B – Limit cycle |
|-----------|-------------------------|-----------------------|----------------------------------|----------------------|
| $k_1$     | 1                       | 1.1994                | 5314.5                           | 4.5915 x 10⁵         |
| $k_2$     | 2                       | 19327                 | 1291                             | 22.28                |
| $k_3$     | 3                       | 0.36953               | 44.965                           | 3.2585 x 10⁶         |
| $k_4$     | 1                       | 29332                 | 9.2497 x 10⁵                     | 9.7962               |
| $k_5$     | 1                       | 4.1166 x 10⁵          | 1.3334                           | 213.71               |
| $k_6$     | 2                       | 552.32                | 2.0451                           | 14.154               |
| $k_{b1}$  | 3                       | 2733.8                | 5241                             | 2531.6               |
| $k_{b2}$  | 3                       | 2.6221 x 10⁶          | 4.0205                           | 1.0435 x 10⁵         |
| $k_{b3}$  | 2                       | 109.37                | 64.271                           | 19464                |
| $k_{b4}$  | 1                       | 50.134                | 54.606                           | 2.4078 x 10⁶         |
| $k_{b5}$  | 4                       | 6.0968                | 2.1496 x 10⁵                     | 8.8294 x 10⁵         |
| $k_{b6}$  | 3                       | 103.34                | 2.7625 x 10⁵                     | 1.0831 x 10⁵         |
| $k_{ub1}$ | 2                       | 85.59                 | 149.19                           | 3208.6               |
| $k_{ub2}$ | 1                       | 7.9504                | 76.203                           | 1.5788 x 10⁶         |
| $k_{ub3}$ | 2                       | 10.303                | 2.2707                           | 209.83               |
| $k_{ub4}$ | 3                       | 1.6602 x 10⁵          | 27238                            | 5.6317 x 10⁵         |
| $k_{ub5}$ | 4                       | 28.243                | 6.0681                           | 785.47               |
| $k_{ub6}$ | 5                       | 0.29981               | 0.40324                          | 22.769               |
| $E_{tot}$ | 0.003                   | 0.0026087             | 0.0012244                        | 0.0068182            |
| $F_{tot}$ | 0.002                   | 0.018996              | 0.0052442                        | 0.0084204            |

Parameter values used to generate the phase plots, bifurcation diagrams, and time courses in Figures 3 through 5.
A  Mixed

\[ E + S_{00} \rightleftharpoons E S_{00} \rightarrow E S_{01} \rightarrow E + S_{11} \]

\[ k_{off} \uparrow \]

\[ k_{cat} \downarrow \]

B  Processive: \( k_{off} \ll k_{cat} \)

\[ E + S_{00} \rightleftharpoons E S_{00} \rightarrow E S_{01} \rightarrow E + S_{11} \]

C  Distributive: \( k_{off} \gg k_{cat} \)

\[ E + S_{00} \rightleftharpoons E S_{00} \rightarrow E S_{01} \rightarrow E + S_{11} \]
