Supporting Material: Cellular compartments cause multistability in biochemical reaction networks and allow cells to process more information

Heather A Harrington*
Division of Molecular Biosciences,
Imperial College London, London, U.K.,

Elisenda Feliu*
Department of Mathematical Sciences, University of Copenhagen,
Copenhagen, Denmark,
*Joint first authors

Carsten Wiuf [1]
Department of Mathematical Sciences, University of Copenhagen,
Copenhagen, Denmark

Michael PH Stumpf[2]
Division of Molecular Biosciences,
Imperial College London, London, U.K.

[1]Corresponding author. Address: Department of Mathematical Sciences, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen, Denmark Tel.: +45 353 20695, Fax: +45 353 20704

[2]Corresponding author. Address: Division of Molecular Biosciences, Imperial College London, Wolfson Building, London, SW7 2AZ, U.K., Tel.: +44(0)2075945280, Fax: +44(0)2075949111

Overview

In the Supporting Material we illustrate the claims made in the main text in more detail.

In Section 1 we study a one-site phosphorylation cycle, which is monostationary, and show that shuttling species can introduce multistationarity. In Section 2 we study the extended two-site phosphorylation cycle. Without compartmentalization the two-site modification cycle exhibits multistationarity for some choices of rate constants but not all. We show that compartmentalization can introduce multistationarity even if the rate contents do not allow multistationarity in a two-site system without compartmentalization.

1 Shuttling in a one-site phosphorylation cycle

Reactions and rate constants. We consider a one-site phosphorylation cycle with species S, S^* (the unphosphorylated and phosphorylated substrates), E (kinase), F (phosphatase), and X, Y (intermediate complexes). Phosphorylation and dephosphorylation are assumed to follow a Michaelis-Menten mechanism (see below and main text). This motif cannot admit multiple steady states and is monostable (1).

To study the effect of compartmentalization we assume that the species S, S^*, E, X can shuttle between the cytoplasm and the nucleus (see Figure 1). We let Z^c denote the species Z in the cytoplasm. Then, we have the following reactions:

- Reactions in the nucleus:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} X \xrightarrow{k_3} E + S^* \qquad F + S^* \underset{k_5}{\overset{k_4}{\rightleftarrows}} Y \xrightarrow{k_6} F + S$$

- Reactions in the cytoplasm:

$$E^c + S^c \underset{k_8}{\overset{k_7}{\rightleftarrows}} X^c \xrightarrow{k_9} E^c + S^c \qquad F^c + S^{c*} \underset{k_{11}}{\overset{k_{10}}{\rightleftarrows}} Y^c \xrightarrow{k_{12}} F^c + S^{c*}$$

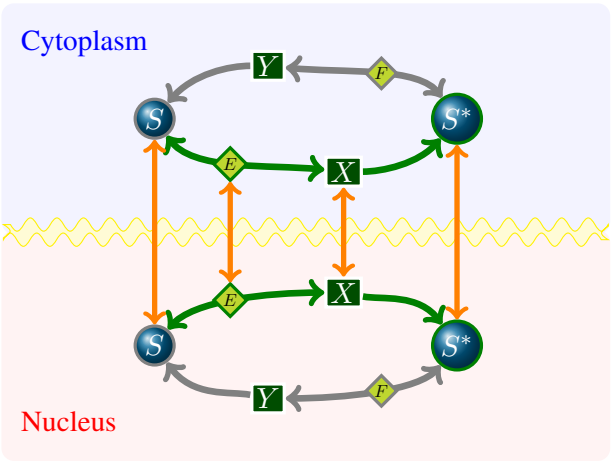

Figure 1: Shuttling of a one-site phosphorylation cycle between the nucleus and the cytoplasm.

- Shuttling reactions:

$$E \underset{k_{17}}{\overset{k_{13}}{\rightleftarrows}} E^c \qquad X \underset{k_{18}}{\overset{k_{14}}{\rightleftarrows}} X^c \qquad S \underset{k_{19}}{\overset{k_{15}}{\rightleftarrows}} S^c \qquad S^* \underset{k_{20}}{\overset{k_{16}}{\rightleftarrows}} S^{c*}$$

To ease the notation, we have changed the notation of the reaction constants k_r in the main text and simply labeled them with consecutive numbers $k_1, \ldots, k_{20}$. The correspondence between the two notations is shown below:

Here	k_1	k_2	k_3	k_4	k_5	k_6	k_7	k_8	k_9	k_{10}
Main text	$k_{\mathrm{on,E}}$	$k_{\mathrm{off,E}}$	$k_{\mathrm{cat,E}}$	$k_{\mathrm{on,F}}$	$k_{\mathrm{off,F}}$	$k_{\mathrm{cat,F}}$	$k^c_{\mathrm{on,E}}$	$k^c_{\mathrm{off,E}}$	$k^c_{\mathrm{cat,E}}$	$k^c_{\mathrm{on,F}}$
Here	k_{11}	k_{12}	k_{13}	k_{14}	k_{15}	k_{16}	k_{17}	k_{18}	k_{19}	k_{20}
Main text	$k^c_{\mathrm{off,F}}$	$k^c_{\mathrm{cat,F}}$	$k_{\mathrm{out,E}}$	$k_{\mathrm{out,X}}$	$k_{\mathrm{out,S}}$	$k_{\mathrm{out,S^*}}$	$k_{\mathrm{in,E}}$	$k_{\mathrm{in,X}}$	$k_{\mathrm{in,S}}$	$k_{\mathrm{in,S^*}}$

Mass-action system of ordinary differential equations. We order the set of species in the following way:

$$(x_1, x_2, x_3, x_4, x_5, x_6) = (E, X, S, S^*, F, Y), \quad (x_7, x_8, x_9, x_{10}, x_{11}, x_{12}) = (E^c, X^c, S^c, S^{c*}, F^c, Y^c).$$

By assuming the law of mass-action, the dynamics of this reaction network is modeled by the following system of ordinary differential equations (reference to time t is omitted, $x_i = x_i(t)$):

$$\begin{aligned}
\dot{x}_1 &= -k_{13}x_1 + k_2x_2 + k_3x_2 - k_1x_1x_3 + k_{17}x_7,\\
\dot{x}_2 &= -k_2x_2 - k_3x_2 - k_{14}x_2 + k_1x_1x_3 + k_{18}x_8,\\
\dot{x}_3 &= k_2x_2 - k_{15}x_3 - k_1x_1x_3 + k_6x_6 + k_{19}x_9,\\
\dot{x}_4 &= k_3x_2 - k_{16}x_4 - k_4x_4x_5 + k_5x_6 + k_{20}x_{10},\\
\dot{x}_5 &= -k_4x_4x_5 + k_5x_6 + k_6x_6,\\
\dot{x}_6 &= k_4x_4x_5 - k_5x_6 - k_6x_6,\\
\dot{x}_7 &= k_{13}x_1 - k_{17}x_7 + k_8x_8 + k_9x_8 - k_7x_7x_9,\\
\dot{x}_8 &= k_{14}x_2 - k_8x_8 - k_9x_8 - k_{18}x_8 + k_7x_7x_9,\\
\dot{x}_9 &= k_{15}x_3 + k_8x_8 - k_{19}x_9 - k_7x_7x_9 + k_{12}x_{12},\\
\dot{x}_{10} &= k_{16}x_4 + k_9x_8 - k_{20}x_{10} - k_{10}x_{10}x_{11} + k_{11}x_{12},\\
\dot{x}_{11} &= -k_{10}x_{10}x_{11} + k_{11}x_{12} + k_{12}x_{12},\\
\dot{x}_{12} &= k_{10}x_{10}x_{11} - k_{11}x_{12} - k_{12}x_{12}.
\end{aligned} \tag{1}$$

This dynamical system has four conservation laws, accounting for the fact that the amounts of enzymes and substrate are conserved:

$$\begin{aligned}
0 &= \dot{x}_1 + \dot{x}_2 + \dot{x}_7 + \dot{x}_8,\\
0 &= \dot{x}_5 + \dot{x}_6,\\
0 &= \dot{x}_2 + \dot{x}_3 + \dot{x}_4 + \dot{x}_6 + \dot{x}_8 + \dot{x}_9 + \dot{x}_{10} + \dot{x}_{12},\\
0 &= \dot{x}_{11} + \dot{x}_{12}.
\end{aligned} \tag{2}$$

These conservation laws can be verified but adding the corresponding equations in (1). Since the model does not incorporate shuttling of the phosphatase, the amount of phosphatase is conserved separately in each compartment.

Let S_{tot} denote the total amount of substrate, and $E_{tot}, F_{tot}, F^c_{tot}$ denote the total amounts of kinase and phosphatase in the system, respectively. The differential equations in (2) lead to the following equations that are fulfilled at any time:

$$\begin{aligned} E_{tot} &= x_1 + x_2 + x_7 + x_8 \\ F_{tot} &= x_5 + x_6 \\ S_{tot} &= x_2 + x_3 + x_4 + x_6 + x_8 + x_9 + x_{10} + x_{12} \\ F^c_{tot} &= x_{11} + x_{12}. \end{aligned} \tag{3}$$

The steady states of the system are obtained by setting all derivatives $\dot{x}_i$ to zero. The system has the *capacity for multiple steady states* if there exist rate constants $k_1, \ldots, k_{20}$ and positive total amounts $S_{tot}, E_{tot}, F_{tot}, F^c_{tot}$ such that the equations $\dot{x}_i = 0$ together with (3) have more than one positive solution. Therefore, for fixed reaction rates and total amounts, determination of multistationarity implies solving a system of polynomial equations in 12 indeterminates (variables). The equations corresponding to the conservation laws are linear, while those corresponding to setting the derivatives to zero are quadratic (that is, they have terms of total degree 1 and 2).

Rate constants and total amounts for multistationarity (for Figs. 2, 3 in the main text). The CRNT toolbox (2) provides a unique set of rate constants for which the system admits multiple positive steady states

$$\begin{array}{lllll} k_1 = 11.679195 & k_2 = 144.94137 & k_3 = 91.527059 & k_4 = 207.26904 & k_5 = 22.115015, \\ k_6 = 309.97808, & k_7 = 49.545796, & k_8 = 8.8750284, & k_9 = 262.90818, & k_{10} = 356.03934, \\ k_{11} = 1.8978202, & k_{12} = 44.457164, & k_{13} = 1.0903408, & k_{14} = 305.42214, & k_{15} = 47.547732, \\ k_{16} = 41.866754, & k_{17} = 86.473107, & k_{18} = 215.67801, & k_{19} = 1, & k_{20} = 165.98446. \end{array}$$

For this set of rate constants, two steady states are provided with total amounts:

$$E_{tot} = 20.7066814, \quad S_{tot} = 35.21053215, \quad F_{tot} = 3.84921092, \quad F^c_{tot} = 11.0903086.$$

We aim to exemplify multistationarity with rate constants that are more biologically reasonable and of the order of experimentally determined values (3, 4). To this end, we have manually investigated the effect of changing a specific rate or a total amount with respect to the emergence of multistationarity. We guide the proposed changes by the structure of the steady-state equations. This procedure has allowed us to tune the rate constants and total amounts to reasonable values without loosing multistationarity. Specifically, we settled for the rates (used to create Figures 2 and 3 in the main text):

$$\begin{array}{lllllll} k_1 = 0.049 & k_2 = 0.009 & k_3 = 0.262 & k_4 = 0.356 & k_5 = 0.002 & k_6 = 0.044 & k_7 = 0.011 \\ k_8 = 0.144 & k_9 = 0.091 & k_{10} = 0.207 & k_{11} = 0.022 & k_{12} = 0.309 & k_{13} = 0.16 & k_{14} = 0.14 \\ k_{15} = 0.001 & k_{16} = 0.166 & k_{17} = 0.0006 & k_{18} = 0.33 & k_{19} = 0.047 & k_{20} = 0.041, & \end{array}$$

and the total amounts $\{E_{tot}, S_{tot}, F_{tot}, F^c_{tot}\} = \{22, 35, 11, 3\}$,where forward kinetic reaction rates ($k_1, k_3, k_4, k_6, k_7, k_9, k_{10}, k_{12}$) have units $\mu M^{-1} s^{-1}$ and all kinetic and shuttling rates have units s^{-1}, which are within an order of magnitude of existing studies (3, 4). With these parameters, there are three steady states, of which two are stable. Specifically, the steady states are approximately:

$$\begin{aligned} SS_1 &= (0.676, 2.357, 16.33, 1.261, 1.023, 9.977, 17.671, 1.296, 2.068, 0.751, 2.041, 0.959) \\ SS_2 &= (0.183, 0.965, 27.220, 0.126, 5.623, 5.377, 20.391, 0.461, 0.559, 0.107, 2.812, 0.188) \\ SS_3 &= (1.062, 2.87, 11.847, 2.145, 0.625, 10.375, 16.371, 1.701, 3.109, 1.503, 1.546, 1.454). \end{aligned}$$

The steady state SS_1 is unstable and has only one eigenvalue with positive real part.

Conditions for monostationarity (Eqns (8) in the main text). Not all choices of rate constants and total amounts have the capacity for multistationarity. We show here that there is a set of *necessary* conditions for the existence of multistationarity that depends exclusively on the shuttling rates. To see this, we apply the Jacobian injectivity criterion to a function that in part consists of the right hand sides of the conservation laws (1).

Specifically, we consider the polynomial function $f_\kappa\colon \mathbb{R}^{12} \to \mathbb{R}^{12}$ given by the right-hand side of the four conservation equations (3) and the equations in (1) for all $\dot{x}_i$ except for $\dot{x}_1, \dot{x}_2, \dot{x}_5$ and $\dot{x}_{11}$. The latter equations are redundant and can be obtained from the conserved equations in (2). The 12 components of the function $f_\kappa = (f_{\kappa,1}, \ldots, f_{\kappa,12})$ are

$$
\begin{aligned}
f_{\kappa,1} &= x_1 + x_2 + x_7 + x_{11},\\
f_{\kappa,2} &= x_2 + x_3 + x_4 + x_6 + x_8 + x_9 + x_{10} + x_{12},\\
f_{\kappa,3} &= x_5 + x_6,\\
f_{\kappa,4} &= x_{11} + x_{12},\\
f_{\kappa,5} &= k_2x_2 - k_{15}x_3 - k_1x_1x_3 + k_6x_6 + k_{19}x_9,\\
f_{\kappa,6} &= k_3x_2 - k_{16}x_4 - k_4x_4x_5 + k_5x_6 + k_{20}x_{10},\\
f_{\kappa,7} &= k_4x_4x_5 - k_5x_6 - k_6x_6,\\
f_{\kappa,8} &= k_{13}x_1 - k_{17}x_7 + k_8x_8 + k_9x_8 - k_7x_7x_9,\\
f_{\kappa,9} &= k_{14}x_2 - k_8x_8 - k_9x_8 - k_{18}x_8 + k_7x_7x_9,\\
f_{\kappa,10} &= k_{15}x_3 + k_8x_8 - k_{19}x_9 - k_7x_7x_9 + k_{12}x_{12},\\
f_{\kappa,11} &= k_{16}x_4 + k_9x_8 - k_{20}x_{10} - k_{10}x_{10}x_{11} + k_{11}x_{12},\\
f_{\kappa,12} &= k_{10}x_{10}x_{11} - k_{11}x_{12} - k_{12}x_{12}.
\end{aligned}
$$

If this function is injective over the real positive numbers $\mathbb{R}^n_+$, then multiple positive steady states with the same total amounts cannot occur. As described in the main text, we use the Jacobian injectivity criterion to investigate conditions on the rate constants for which the function is injective. Since f_κ is quadratic, the criterion applies. The determinant of the Jacobian of f_κ can be computed using any software that enables algebraic (symbolic) computations, like Mathematica or Maple. We compute the determinant and extract the coefficients. These coefficients are polynomials in the rate constants and most of them contain only positive summands. Therefore, we search for the coefficients that have negative summands. After appropriate factorization and simplification, we conclude that the coefficients are all positive if and only if the following expressions are positive:

$$
\begin{aligned}
C_1 =& k_9k_{14} + k_9k_{17} + k_3(k_{18} - k_{17}) = k_9k_{14} + (k_9 - k_3)k_{17} + k_3k_{18},\\
C_2 =& k_3k_{12}(k_{15} - k_{16})(k_{18} - k_{17}) + k_3k_{15}k_{16}(k_{18} - k_{17}) + k_{12}k_{15}k_{16}k_{18} + k_9k_{14}k_{15}k_{16}\\
&+ k_{12}k_{14}k_{15}k_{16} + k_{12}k_{14}k_{16}k_{17} + k_9k_{15}k_{16}k_{17} + k_{12}k_{16}k_{17}k_{18},\\
C_3 =& k_3k_{12}k_{15}(k_{18} - k_{17}) + k_6k_9k_{14}k_{15} + k_6k_{12}k_{14}k_{15} + k_6k_{12}k_{14}k_{17} + k_6k_9k_{15}k_{17}\\
&+ k_6k_{12}k_{15}k_{18} + k_6k_{12}k_{17}k_{18},\\
C_4 =& k_3k_{15}(k_{18} - k_{17})k_{20} + k_6k_9k_{14}k_{15} + k_6k_9k_{15}k_{17} + k_6k_9k_{14}k_{20} + k_6k_{14}k_{15}k_{20}\\
&+ k_9k_{14}k_{15}k_{20} + k_6k_9k_{17}k_{20} + k_6k_{14}k_{17}k_{20} + k_9k_{15}k_{17}k_{20} + k_6k_{15}k_{18}k_{20} + k_6k_{17}k_{18}k_{20},
\end{aligned}
$$

$$
\begin{aligned}
C_5 =& k_3k_{13} + k_9(k_{14} - k_{13}) + k_3k_{18} = (k_3 - k_9)k_{13} + k_9k_{14} + k_3k_{18},\\
C_6 =& k_6k_9(k_{14} - k_{13})k_{19} + k_6k_{12}k_{13}k_{14} + k_6k_{12}k_{13}k_{18} + k_3k_{12}k_{13}k_{19} + k_6k_{12}k_{14}k_{19}\\
&+ k_3k_{12}k_{18}k_{19} + k_6k_{12}k_{18}k_{19},\\
C_7 =& k_9(k_{14} - k_{13})k_{16}k_{19} + k_3k_{12}k_{13}k_{16} + k_{12}k_{13}k_{14}k_{16} + k_3k_{12}k_{16}k_{18} + k_{12}k_{13}k_{16}k_{18}\\
&+ k_3k_{12}k_{13}k_{19} + k_3k_{13}k_{16}k_{19} + k_{12}k_{14}k_{16}k_{19} + k_3k_{12}k_{18}k_{19} + k_3k_{16}k_{18}k_{19} + k_{12}k_{16}k_{18}k_{19},\\
C_8 =& k_6k_9(k_{14} - k_{13})(k_{19} - k_{20}) + k_9(k_{14} - k_{13})k_{19}k_{20} + k_6k_{13}k_{14}k_{20} + k_6k_{13}k_{18}k_{20}\\
&+ k_3k_{13}k_{19}k_{20} + k_6k_{14}k_{19}k_{20} + k_3k_{18}k_{19}k_{20} + k_6k_{18}k_{19}k_{20}.
\end{aligned}
$$

Observe that these expressions *only* involve the 8 shuttling rates and k_3, k_6, k_9, k_{12}. Instances for which the coefficients $C_1, \ldots, C_8$ are negative exist. If

$$k_{20} \leq k_{19}, \quad k_{18} \geq k_{17}, \quad k_{16} \leq k_{15}, \quad k_{14} \geq k_{13}, \tag{4}$$

then $C_i > 0$ for all i and hence multistationarity cannot occur for any choice of total amounts (these inequalities correspond to Eqns. (8) in the main text). However, there is no guarantee that when these conditions fail, the system admits multiple steady states for some total amounts. Figure 1 above is reproduced as Figure 2 with the shuttling rate constants indicated.

We assume now that the dissociation constants are the same in the two compartments (the nucleus and the cytoplasm), that is, we assume that $k_3 = k_9$ and $k_6 = k_{12}$. In this case $C_i > 0$ for all $i \neq 2, 8$ and all shuttling rate constants. Therefore, two conditions suffice to guarantee monostationarity, namely:

$$
\begin{aligned}
\widetilde{C}_2 =& k_9k_{12}(k_{15} - k_{16})(k_{18} - k_{17}) + k_9k_{14}k_{15}k_{16} + k_{12}k_{14}k_{15}k_{16}\\
&+ k_{12}k_{14}k_{16}k_{17} + k_9k_{15}k_{16}k_{18} + k_{12}k_{15}k_{16}k_{18} + k_{12}k_{16}k_{17}k_{18} > 0,\\
\widetilde{C}_8 =& k_9k_{12}(k_{14} - k_{13})(k_{19} - k_{20}) + k_{12}k_{13}k_{14}k_{20} + k_{12}k_{13}k_{18}k_{20}\\
&+ k_9k_{14}k_{19}k_{20} + k_{12}k_{14}k_{19}k_{20} + k_9k_{18}k_{19}k_{20} + k_{12}k_{18}k_{19}k_{20} > 0.
\end{aligned}
$$

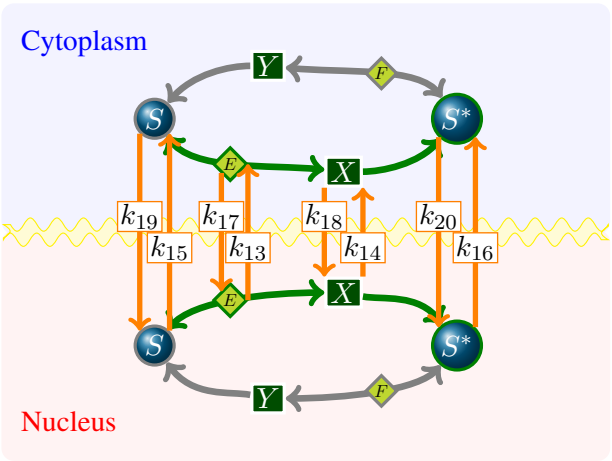

Figure 2: Shuttling rates for the one-site phosphorylation cycle

The first corresponds to C_2 and the second to C_8. By inspection of these two expressions, we conclude that multistationarity cannot occur in any of the following cases:

$$\begin{aligned}
&\text{(i)} && k_{20} \leq k_{19}, && k_{18} \geq k_{17}, && k_{16} \leq k_{15}, && k_{14} \geq k_{13},\\
&\text{(ii)} && k_{20} \geq k_{19}, && k_{18} \geq k_{17}, && k_{16} \leq k_{15}, && k_{14} \leq k_{13},\\
&\text{(iii)} && k_{20} \leq k_{19}, && k_{18} \leq k_{17}, && k_{16} \geq k_{15}, && k_{14} \geq k_{13},\\
&\text{(iv)} && k_{20} \geq k_{19}, && k_{18} \leq k_{17}, && k_{16} \geq k_{15}, && k_{14} \leq k_{13}.
\end{aligned}$$

Note that these *only* involve the rate constants for the shuttling reactions. If the dissociation rate constants are not exactly the same in the cytoplasm and in the nucleus, but very similar, then the conditions above are still sufficient.

We see that the rate constants go in pairs: the shuttling rate constants of S relate to those of S^*, and the shuttling rate constants of E to those of X. In particular, the following conditions are necessary for multistationarity:

(1) If X shuttles into the nucleus slower than E then S shuttles into the cytoplasm slower than S^* and vice versa.

(2) If X shuttles into the cytoplasm slower than E then S shuttles into the nucleus slower than S^* and vice versa.

Sets of rate constants for which $I_1 < 0$ can for instance be obtained by letting the product k_9k_{16} be large and the remaining products be small such that $k_{14}k_{15} + k_{12}k_{17} - k_{12}k_{18} + k_{15}k_{18} < 0$ is satisfied.

#	No multistationarity	Multistationarity
1	All	None
2	$\{S,S^*\}$ $\{E,Y\}$ $\{F,X\}$ $\{S^*,E\}$ $\{S,F\}$ $\{S,X\}$ $\{S^*,Y\}$ $\{E,X\}$ $\{F,Y\}$ $\{S,E\}$ $\{S^*,F\}$	$\{E,F\}$ $\{X,Y\}$ $\{S^*,X\}$ $\{S,Y\}$
3	$\{X,E,F\}$ $\{Y,E,F\}$ $\{X,Y,E\}$ $\{X,Y,F\}$ $\{S,F,X\}$ $\{S^*,E,Y\}$ $\{S,E,F\}$ $\{S^*,F,E\}$	$\{S,E,X\}$ $\{S^*,F,Y\}$ $\{S,E,Y\}$ $\{S^*,E,X\}$ $\{S^*,F,X\}$ $\{S,E,S^*\}$ $\{S^*,F,S\}$ $\{S,S^*,X\}$ $\{S,X,Y\}$ $\{S^*,Y,X\}$ $\{S,F,Y\}$ $\{S,S^*,Y\}$
4	$\{Y,X,E,F\}$	$\{S,S^*,X,F\}$ $\{S,S^*,Y,E\}$ $\{S,E,X,Y\}$ $\{S^*,F,X,Y\}$ $\{S,F,X,Y\}$ $\{S^*,E,X,Y\}$ $\{S,S^*,X,Y\}$ $\{S,S^*,E,F\}$ $\{S,E,F,X\}$ $\{S^*,E,F,Y\}$ $\{S,E,F,Y\}$ $\{S^*,E,F,X\}$ $\{S,S^*,X,E\}$ $\{S,S^*,Y,F\}$
5, 6	None	All

Table 1: One-site phosphorylation system. For all possible sets of shuttling species it is indicated if the system has the capacity for multiple steady states or not.

Sets of shuttling species and multistationarity. We have shown that if the species E, X, S, S^* shuttle between compartments, multistationarity is created. We next investigate what the sets of shuttling species that provide multistationarity are. The results are summarized in Table 1. We use a systematic way to classify each motif: First, we check if the system fulfills the Jacobian injectivity criterion for all rate constants. If the coefficients of the polynomial in x given by the determinant of the Jacobian (as above) are all positive, then the system cannot exhibit multistationarity for any set of total amounts (see also (5)). If the criterion fails then we use the CRNT toolbox.

We have obtained that if only one species shuttles then multistationarity cannot occur. That is, at least two species, e.g. $\{S^*, X\}$ or $\{S, Y\}$, are required to create multistationarity in the one-site phosphorylation cycle for certain total amounts and rate constants. The addition of shuttling species maintains multistationarity.

Effects of varying the shuttling rates. We analyze the steady-state response of S^* in the nucleus as the shuttling rate constants and total amounts change in the system.

The following table summarizes the type of saddle-node bifurcation curves obtained as shuttling rate constants of molecular species are varied.

Rate constant	Rate-response curve
$k_{13}, k_{14}, k_{19}, k_{20}$	For large rate constant, only a low stable steady state is obtained
k_{15}, k_{17}, k_{18}	For a small rate constant, only a high stable steady state is obtained
k_{16}	Similar to the previous case, but the high branch decreases (Fig. S1).

By varying a total amount and shuttling rate constant simultaneously, the system may undergo irreversible switches. This occurs with respect to shuttling rate constants k_{14}, k_{15}, k_{17}, and k_{20}. Specifically, for shuttling parameters, k_{15} and k_{17}, the irreversible switch is obtained by either increasing F_{tot} or decreasing E_{tot}, S_{tot} or F^c_{tot}. As the value of the shuttling rate constant increases, the response curve switches from a low to a high steady state, favoring accumulation in the nucleus. Conversely, increasing the value of shuttling parameters k_{14} and k_{20} induces an irreversible switch from the high to low steady-state by either decreasing F_{tot} or increasing E_{tot}, S_{tot}. As highlighted in the main text (see Figure 3), the k_{20} bifurcation is irreversible at baseline parameter values.

2 Shuttling in a two-site phosphorylation cycle

In eukaryotes, most protein phosphorylation events take place in more than one site. It is well known that multisite phosphorylation can cause multistationarity by itself (6, 7). However, multistationarity does not occur for all choices of rate constants.

We next investigate the effect of adding species compartmentalization in a two-site (sequential) phosphorylation system. We first determine rate constants for which the two-site system cannot exhibit multistationarity. Then, we add species shuttling and determine shuttling rate constants that induce multistationarity.

Conditions for monostationarity in a two-site phosphorylation cycle. We consider a two-site phosphorylation cycle in which modifications take place sequentially. The reactions describing the system are:

$$S_0 + E \underset{k_2}{\overset{k_1}{\rightleftarrows}} X_1 \xrightarrow{k_3} S_1 + E \qquad S_1 + E \underset{k_5}{\overset{k_4}{\rightleftarrows}} X_2 \xrightarrow{k_6} S_2 + E$$

$$S_1 + F \underset{k_8}{\overset{k_7}{\rightleftarrows}} Y_1 \xrightarrow{k_9} S_0 + F \qquad S_2 + F \underset{k_{11}}{\overset{k_{10}}{\rightleftarrows}} Y_2 \xrightarrow{k_{12}} S_1 + F$$

The set of species is ordered such that

$$(x_1, \ldots, x_9) = (E, X_1, S_0, S_1, F, Y_1, S_2, X_2, Y_2).$$

Assuming mass-action kinetics, then the differential equations describing the dynamics of the species concentrations are:

$$
\begin{aligned}
\dot{x_1} &= k_2x_2 + k_3x_2 - k_1x_1x_3 - k_4x_1x_4 + k_5x_8 + k_6x_8,\\
\dot{x_2} &= -k_2x_2 - k_3x_2 + k_1x_1x_3,\\
\dot{x_3} &= k_2x_2 - k_1x_1x_3 + k_9x_6,\\
\dot{x_4} &= k_3x_2 - k_4x_1x_4 - k_7x_4x_5 + k_8x_6 + k_5x_8 + k_{12}x_9,\\
\dot{x_5} &= -k_7x_4x_5 + k_8x_6 + k_9x_6 - k_{10}x_5x_7 + k_{11}x_9 + k_{12}x_9,\\
\dot{x_6} &= k_7x_4x_5 - k_8x_6 - k_9x_6,\\
\dot{x_7} &= -k_{10}x_5x_7 + k_6x_8 + k_{11}x_9,\\
\dot{x_8} &= k_4x_1x_4 - k_5x_8 - k_6x_8,\\
\dot{x_9} &= k_{10}x_5x_7 - k_{11}x_9 - k_{12}x_9.
\end{aligned}
$$

This system has the following conserved amounts:

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

The steady-state equations are given by $\dot{x}_i = 0$. Because of the constraints given by the conservation laws, the equations $\dot{x}_1 = 0$, $\dot{x}_2 = 0$ and $\dot{x}_5 = 0$ are redundant and can be removed.

We proceed as above to determine rate constants for which the system cannot have multiple steady states. That is, we apply the Jacobian injectivity criterion. We consider the function $f_\kappa \colon \mathbb{R}^9 \to \mathbb{R}^9$ given by the three equations coming from the conservation laws and the 6 remaining steady-state equations:

$$
\begin{aligned}
f_{\kappa,1}(x) &= x_1 + x_2 + x_8,\\
f_{\kappa,2}(x) &= x_2 + x_3 + x_4 + x_6 + x_7 + x_8 + x_9,\\
f_{\kappa,3}(x) &= x_5 + x_6 + x_9,\\
f_{\kappa,4}(x) &= k_2x_2 - k_1x_1x_3 + k_9x_6,\\
f_{\kappa,5}(x) &= k_3x_2 - k_4x_1x_4 - k_7x_4x_5 + k_8x_6 + k_5x_8 + k_{12}x_9,\\
f_{\kappa,6}(x) &= k_7x_4x_5 - k_8x_6 - k_9x_6,\\
f_{\kappa,7}(x) &= -k_{10}x_5x_7 + k_6x_8 + k_{11}x_9,\\
f_{\kappa,8}(x) &= k_4x_1x_4 - k_5x_8 - k_6x_8,\\
f_{\kappa,9}(x) &= k_{10}x_5x_7 - k_{11}x_9 - k_{12}x_9.
\end{aligned}
$$

If this function is injective over the positive real numbers then multiple positive steady states cannot occur with the same total amounts in the conservation laws. Next, we compute the determinant of the Jacobian of f_κ. As a polynomial in x, the only coefficients (which depend on the rate constants k_i) of the determinant that are not sums of positive terms are:

$$
\begin{aligned}
C_1 &= -k_2k_4k_6k_7k_9k_{10} - k_3k_4k_6k_7k_9k_{10} - k_1k_4k_6k_7k_9k_{11} - k_1k_4k_6k_7k_9k_{12}\\
&\quad + k_1k_3k_5k_7k_{10}k_{12} + k_1k_3k_6k_7k_{10}k_{12} + k_1k_3k_4k_8k_{10}k_{12} + k_1k_3k_4k_9k_{10}k_{12}\\
C_2 &= -k_1k_4k_7k_{10}(k_6k_9 - k_3k_{12}).
\end{aligned}
$$

If a choice of rate constants fulfills $C_1, C_2 > 0$, then the system cannot have multiple positive steady states for any total amounts. The coefficient C_1 can be rewritten as

$$C_1 = -k_6k_9k_4k_7(k_2k_{10} + k_3k_{10} + k_1k_{11} + k_1k_{12}) + k_3k_{12}k_1k_{10}(k_5k_7 + k_6k_7 + k_4k_8 + k_4k_9).$$

For $C_2 > 0$ we require

$$k_3/k_6 > k_9/k_{12}.$$

If this inequality is fulfilled then $C_1 > 0$ if also

$$k_4k_7(k_2k_{10} + k_3k_{10} + k_1k_{11} + k_1k_{12}) < k_1k_{10}(k_5k_7 + k_6k_7 + k_4k_8 + k_4k_9).$$

This inequality can be rewritten as

$$\frac{k_2 + k_3}{k_1} + \frac{k_{11} + k_{12}}{k_{10}} < \frac{k_5 + k_6}{k_4} + \frac{k_8 + k_9}{k_7}.$$

Let

$$\alpha_1 = \frac{k_5 + k_6}{k_4} - \frac{k_2 + k_3}{k_1}, \qquad \alpha_2 = \frac{k_8 + k_9}{k_7} - \frac{k_{11} + k_{12}}{k_{10}}, \qquad \alpha_3 = \frac{k_3}{k_6} - \frac{k_9}{k_{12}}.$$

If

$$\alpha_1, \alpha_2, \alpha_3 > 0, \tag{5}$$

then the two-site phosphorylation system cannot have multiple positive steady states no matter the values of the total amounts. That is, a sufficient condition for the preclusion of multistationarity is obtained.

Observe that α_1 and α_2 imply an inequality between the Michaelis-Menten constants of the kinase and the phosphatase in each phosphorylation site. Namely, the Michaelis-Menten constant of E for the second site is larger than that for the first phosphorylation site, and the Michaelis-Menten constant of F for the first site is larger than that for the second site.

Negation of this condition is *a priori* not sufficient to guarantee multistationarity. However, if $\alpha_3 < 0$, then there exist total amounts for which the motif exhibits multistationarity (8).

Mass-action system of ordinary differential equations for the two-site shuttling. Consider now two copies of a two-site phosphorylation cycle as above and let $S_0, S_1, S_2, X_1, X_2, E$ shuttle between the nucleus and the cytoplasm. The reactions describing the system are:

- Reactions in the nucleus:

$$S_0 + E \underset{k_2}{\overset{k_1}{\rightleftarrows}} X_1 \xrightarrow{k_3} S_1 + E \qquad S_1 + E \underset{k_5}{\overset{k_4}{\rightleftarrows}} X_2 \xrightarrow{k_6} S_2 + E$$

$$S_1 + F \underset{k_8}{\overset{k_7}{\rightleftarrows}} Y_1 \xrightarrow{k_9} S_0 + F \qquad S_2 + F \underset{k_{11}}{\overset{k_{10}}{\rightleftarrows}} Y_2 \xrightarrow{k_{12}} S_1 + F$$

- Reactions in the cytoplasm:

$$S_0^c + E^c \underset{k_{14}}{\overset{k_{13}}{\rightleftarrows}} X_1^c \xrightarrow{k_{15}} S_1^c + E^c \qquad S_1^c + E^c \underset{k_{17}}{\overset{k_{16}}{\rightleftarrows}} X_2^c \xrightarrow{k_{18}} S_2^c + E^c$$

$$S_1^c + F^c \underset{k_{20}}{\overset{k_{19}}{\rightleftarrows}} Y_1^c \xrightarrow{k_{21}} S_0^c + F^c \qquad S_2^c + F^c \underset{k_{23}}{\overset{k_{22}}{\rightleftarrows}} Y_2^c \xrightarrow{k_{24}} S_1^c + F^c$$

- Shuttling reactions:

$$E \underset{k_{31}}{\overset{k_{25}}{\rightleftarrows}} E^c \qquad X_1 \underset{k_{32}}{\overset{k_{26}}{\rightleftarrows}} X_1^c \qquad S_0 \underset{k_{33}}{\overset{k_{27}}{\rightleftarrows}} S_0^c$$

$$S_1 \underset{k_{34}}{\overset{k_{28}}{\rightleftarrows}} S_1^c \qquad S_2 \underset{k_{35}}{\overset{k_{29}}{\rightleftarrows}} S_2^c \qquad X_2 \underset{k_{36}}{\overset{k_{30}}{\rightleftarrows}} X_2^c$$

The species are ordered as:

$$(x_1, \ldots, x_9) = (E, X_1, S_0, S_1, F, Y_1, S_2, X_2, Y_2)$$

$$(x_{10}, \ldots, x_{18}) = (E^c, X_1^c, S_0^c, S_1^c, F^c, Y_1^c, S_2^c, X_2^c, Y_2^c).$$

Assuming mass-action kinetics, the system of differential equations describing the dynamics of the concentrations of the species is:

$$\begin{aligned}
\dot{x}_1 &= k_2x_2 + k_3x_2 - k_1x_1x_3 - k_4x_1x_4 + k_5x_8 + k_6x_8 - k_{25}x_1 + k_{31}x_{10},\\
\dot{x}_2 &= -k_2x_2 - k_3x_2 + k_1x_1x_3 - k_{26}x_2 + k_{32}x_{11},\\
\dot{x}_3 &= k_2x_2 - k_1x_1x_3 + k_9x_6 - k_{27}x_3 + k_{33}x_{12},\\
\dot{x}_4 &= k_3x_2 - k_4x_1x_4 - k_7x_4x_5 + k_8x_6 + k_5x_8 + k_{12}x_9 - k_{28}x_4 + k_{34}x_{13},\\
\dot{x}_5 &= -k_7x_4x_5 + k_8x_6 + k_9x_6 - k_{10}x_5x_7 + k_{11}x_9 + k_{12}x_9,\\
\dot{x}_6 &= k_7x_4x_5 - k_8x_6 - k_9x_6,\\
\dot{x}_7 &= -k_{10}x_5x_7 + k_6x_8 + k_{11}x_9 - k_{29}x_7 + k_{35}x_{16},\\
\dot{x}_8 &= k_4x_1x_4 - k_5x_8 - k_6x_8 - k_{30}x_8 + k_{36}x_{17},\\
\dot{x}_9 &= k_{10}x_5x_7 - k_{11}x_9 - k_{12}x_9,\\
\dot{x}_{10} &= k_{14}x_{11} + k_{15}x_{11} - k_{13}x_{10}x_{12} - k_{16}x_{10}x_{13} + k_{17}x_{17} + k_{18}x_{17} + k_{25}x_1 - k_{31}x_{10},\\
\dot{x}_{11} &= -k_{14}x_{11} - k_{15}x_{11} + k_{13}x_{10}x_{12} + k_{26}x_2 - k_{32}x_{11},\\
\dot{x}_{12} &= k_{14}x_{11} - k_{13}x_{10}x_{12} + k_{21}x_{15} + k_{27}x_3 - k_{33}x_{12},\\
\dot{x}_{13} &= k_{15}x_{11} - k_{16}x_{10}x_{13} - k_{19}x_{13}x_{14} + k_{20}x_{15} + k_{17}x_{17} + k_{24}x_{18} + k_{28}x_4 - k_{34}x_{13},\\
\dot{x}_{14} &= -k_{19}x_{13}x_{14} + k_{20}x_{15} + k_{21}x_{15} - k_{22}x_{14}x_{16} + k_{23}x_{18} + k_{24}x_{18},\\
\dot{x}_{15} &= k_{19}x_{13}x_{14} - k_{20}x_{15} - k_{21}x_{15},\\
\dot{x}_{16} &= -k_{22}x_{14}x_{16} + k_{18}x_{17} + k_{23}x_{18} + k_{29}x_7 - k_{35}x_{16},\\
\dot{x}_{17} &= k_{16}x_{10}x_{13} - k_{17}x_{17} - k_{18}x_{17} + k_{30}x_8 - k_{36}x_{17},\\
\dot{x}_{18} &= k_{22}x_{14}x_{16} - k_{23}x_{18} - k_{24}x_{18}.
\end{aligned}$$

This system has the following conserved amounts:

$$\begin{aligned}
E_{tot} &= x_1 + x_2 + x_8 + x_{10} + x_{11} + x_{17},\\
F_{tot} &= x_5 + x_6 + x_9,\\
F^c_{tot} &= x_{14} + x_{15} + x_{18},\\
S_{tot} &= x_2 + x_3 + x_4 + x_6 + x_7 + x_8 + x_9 + x_{11} + x_{12} + x_{13} + x_{15} + x_{16} + x_{17} + x_{18}.
\end{aligned}$$

Creation of multistationarity in a two-site phosphorylation cycle. We use the CRNT toolbox to obtain initial rate constants and total amounts for which the system admits multiple positive steady states.

The output rate constants do not fulfill $\alpha_1, \alpha_2, \alpha_3 > 0$ in each compartment independently. Hence, it is not possible to decide whether multistationarity arises due to shuttling or due to phosphorylation of two different sites.

Next we investigate the effect of changing the rate constants with respect to the existence of multistationarity for the same total amounts. We proceed by manually modifying the rates while keeping multistationarity and such that the sufficient conditions for the preclusion of multistationarity in a two-site phosphorylation cycle are satisfied in each compartment.

To demonstrate multistationarity with this system, we arbitrarily choose the following rate constants:

- Reaction rates in the nucleus:

$$\begin{array}{llllll} k_1 = 100 & k_2 = 2 & k_3 = 10 & k_4 = 80 & k_5 = 6 & k_6 = 6 \\ k_7 = 350 & k_8 = 3 & k_9 = 10 & k_{10} = 650 & k_{11} = 8 & k_{12} = 8. \end{array}$$

- Reaction rates in the cytoplasm:

$$\begin{array}{llllll} k_{13} = 300 & k_{14} = 1 & k_{15} = 10 & k_{16} = 50 & k_{17} = 1 & k_{18} = 1 \\ k_{19} = 350 & k_{20} = 30 & k_{21} = 190 & k_{22} = 150 & k_{23} = 2 & k_{24} = 20. \end{array}$$

- Shuttling rates:

$$\begin{array}{llllll} k_{25} = 10 & k_{26} = 30 & k_{27} = 70 & k_{28} = 30 & k_{29} = 1 & k_{30} = 10 \\ k_{31} = 450 & k_{32} = 20 & k_{33} = 20 & k_{34} = 25 & k_{35} = 10 & k_{36} = 100. \end{array}$$

This choice of rate constants fulfills that $\alpha_1, \alpha_2, \alpha_3 > 0$ in the nucleus and in the cytoplasm (where in the later, indices of the rate constants in α_* are shifted by 12). Therefore, with these rate constants, the two-site phosphorylation cycles in the nucleus and in the cytoplasm cannot have multiple positive steady states independently of each other.

The system with the shuttling reactions, however, does have the capacity for multistationarity. Specifically, if the total amounts are set to:

$$E_{tot} = 50, \qquad S_{tot} = 100 \qquad F_{tot} = 15 \qquad F^c_{tot} = 21,$$

then the system has three positive steady states: two of them are stable and one is unstable. The positive steady states are the following:

$SS_1 = (1.89, 9.18, 0.62, 1.88, 0.01, 0.7, 25.11, 23.21, 14.28, 0.07, 13.38, 6.8, 0.04, 18.49, 1.15, 0.01, 2.28, 1.36)$
$SS_2 = (6.45, 5.93, 0.1, 0.61, 0.01, 0.17, 35.4, 25.64, 14.82, 0.13, 9.33, 2.8, 0.03, 18.32, 0.8, 0.02, 2.52, 1.89)$
$SS_3 = (0.37, 17.59, 5.96, 2.3, 0.17, 10.66, 0.6, 5.65, 4.16, 0.04, 25.8, 24.89, 0.06, 19.22, 1.72, 0.00044, 0.56, 0.06).$

Here SS_1 is the unstable steady state.

Rate constants to obtain seven steady states (for Figs. 4(B-D) in the main text). Additionally, we have observed that, with specific choices of rate constants and total amounts, up to 7 steady states can be

created in this system. These were arbitrarily chosen for illustration of the possible behavior the system can emit. For instance, consider the rate constants (used in the main text to create Figures 4(B-D)):

$$(k_1, \ldots, k_{12}) = (101, 2, 11, 79, 6, 6, 568, 3, 12, 1502, 8, 8),$$
$$(k_{13}, \ldots, k_{24}) = (210, 3, 34, 49, 1, 1, 344, 26, 187, 149, 1, 1),$$
$$(k_{25}, \ldots, k_{36}) = (10, 34, 7.5, 312.5, 0.1, 0.1, 44, 23, 2, 250, 0.1, 0.1),$$

and the total amounts $(E_{tot}, S_{tot}, F_{tot}, F^c_{tot}) = (57, 111, 15, 21)$. This system has 7 steady states, 4 of which are stable:

$SS_1 = (18.27, 2.41, 0.04, 0.03, 5.05, 6.28, 0.01, 4.12, 3.67, 5.2, 1.45, 0.005, 0.21, 0.01, 0.002, 46.2, 25.61, 20.99)$
$SS_2 = (12.66, 1.2, 0.03, 0.17, 0.47, 3.11, 0.26, 14.67, 11.42, 3.4, 0.73, 0.004, 0.3, 0.01, 0.004, 33.77, 24.34, 20.99)$
$SS_3 = (13.01, 0.05, 0.0009, 0.23, 0.01, 0.09, 16.34, 19.9, 14.9, 2.96, 0.06, 0.003, 0.29, 0.02, 0.01, 17.14, 21.06, 20.1)$
$SS_4 = (13.66, 2.04, 0.004, 0.23, 0.004, 0.039, 35.84, 20.54, 14.96, 2.63, 3.95, 0.3, 0.22, 2.48, 0.84, 0.1, 14.18, 17.76)$
$SS_5 = (14.56, 3.53, 0.006, 0.22, 0.004, 0.03, 40.64, 20.63, 14.96, 2.49, 6.85, 0.56, 0.14, 6.54, 1.45, 0.03, 8.95, 13.01)$
$SS_6 = (4, 15.03, 0.4, 0.49, 0.27, 5.07, 0.38, 12.88, 9.65, 0.12, 23.78, 35.81, 0.2, 14.89, 4.88, 0.001, 1.19, 1.23)$
$SS_7 = (5.94, 11.14, 0.15, 0.5, 0.02, 0.43, 6.89, 19.51, 14.55, 0.12, 18.91, 31.22, 0.17, 14.86, 4.07, 0.002, 1.38, 2.07)$

The stable steady states are SS_1, SS_3, SS_5, SS_6.

As E_{tot} varies, the number of states changes as shown on a log-log scale in Figure S2, corresponding to the bifurcation diagram (Figure 4B in the main text, semi-log scale).

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