SI Text

This supporting information text provides the differential equations that describe the mechanism for robust signaling (Fig. 2b). We present the analytic solution for their fixed points, and study their stability both analytically and numerically. We also analyze the effects of seven additional reactions on input-output robustness. In addition, we compare the present mechanism to the engineering principle of integral feedback (1), and discuss similarities and differences with the example of robust adaptation observed in bacterial chemotaxis (2). We analyze the response time of the system, and present numerical results on the system dynamics for a large set of parameters. Finally, we show that in a previous model of the EnvZ/OmpR system (3) the output is proportional to the ATP concentration.

The differential equations induced by the mechanism for robust signaling:

The reactions and rate constants are as follows:

$$
[1'] \qquad
\begin{aligned}
&(a) \quad \mathrm{X} + \mathrm{T} \underset{k'_1}{\overset{k_1}{\rightleftharpoons}} \mathrm{A} \xrightarrow{v_a(s)} \mathrm{X_P} + \mathrm{D} \\
&(b) \quad \mathrm{X_P} + \mathrm{Y} \underset{k'_2}{\overset{k_2}{\rightleftharpoons}} \mathrm{B} \xrightarrow{v_k} \mathrm{X} + \mathrm{Y_P} \\
&(c) \quad \mathrm{A} + \mathrm{Y_P} \underset{k'_3}{\overset{k_3}{\rightleftharpoons}} \mathrm{C} \xrightarrow{v_p} \mathrm{A} + \mathrm{Y} + \mathrm{P_i}
\end{aligned}
\quad ,
$$

where the symbol T denotes ATP and D denotes ADP. X and X_P denote un-phosphorylated and phosphorylated sensor, respectively. Y and Y_P denote un-phosphorylated and phosphorylated response regulator, respectively. The symbol A denotes the complex X·T, B denotes the complex X_P·Y and C denotes the complex X·T·Y_P. In the reaction pairs of Eqs. **1'a**, **1'b** and **1'c**, the forward rate constants are k_1, k_2 and k_3, respectively, and the backward rate constants are k'_1, k'_2 and k'_3, respectively. $v_a(s)$ is the rate constant in the sensor autophosphorylation reaction **1'a**. It depends on the level s of the input signal. v_k and v_p are the rate constants in the phosphotransfer and dephoshorylation reactions **1'b** and **1'c**.

The total concentrations X_T and Y_T of the sensor and the response regulator proteins, respectively, are assumed to be constant on the time-scale of approach to steady state. This is reasonable since the response time of the system is typically on the order of seconds to minutes, much faster than changes in total protein levels due to protein production or dilution/degradation (4):

[1] $$X + X_P + A + B + C = X_T,$$

[2] $$Y + Y_P + B + C = \mathrm{Y}_T.$$

Similarly, the ATP concentration T is also assumed to be constant. Note that X_T, Y_T and T typically vary from cell to cell.

We use mass-action kinetics to describe the time course of the concentrations of the components in the system. This results in seven first-order, non-linear differential equations:

[3] $$\frac{dA}{dt} = k_1 XT - (k'_1 + v_a)A - k_3 A Y_P + (k'_3 + v_p)C$$

[4] $$\frac{dX_P}{dt} = v_a A - k_2 X_P Y + k'_2 B$$

[5] $$\frac{dY}{dt} = k'_2 B - k_2 X_P Y + v_p C$$

[6] $$\frac{dB}{dt} = k_2 X_P Y - (k'_2 + v_k)B$$

[7] $$\frac{dX}{dt} = k'_1 A - k_1 XT + v_k B$$

[8] $$\frac{dY_P}{dt} = v_k B - k_3 A Y_P + k'_3 C$$

[9] $$\frac{dC}{dt} = k_3 A Y_P - (k'_3 + v_p)C$$

Eqs. **3–9** obey the conservation laws **1** and **2**, which can be used to eliminate two of the seven variables. Thus, the dynamic behavior of the system can be determined by specifying X_T and Y_T together with the initial values of five out of the seven concentrations, utilizing Eqs. **1** and **2** to obtain the initial values of the remaining concentrations and integrating Eqs. **3–9**.

Steady state solutions:

Solving Eqs. **3–9** at steady state shows that the equations have two fixed points, which are denoted F and F^*:

[10] $$F = \begin{pmatrix} Y_P \\ X_P \\ A \\ B \\ C \\ Y \\ X \end{pmatrix} = \begin{pmatrix} Y_T \\ X_T \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \end{pmatrix},$$

[11] $$F^* = \begin{pmatrix} Y_P^* \\ X_P^* \\ A^* \\ B^* \\ C^* \\ Y^* \\ X^* \end{pmatrix} = \begin{pmatrix} \frac{k'_3 + v_p}{k_3} \frac{v_a(s)}{v_p} \\ \frac{1}{2}\left(-\eta + \sqrt{\eta^2 - 4\sigma}\right) \\ \frac{\beta}{\alpha\gamma}(X_T - X_P^*) \\ \frac{v_a}{v_k} A^* \\ \frac{v_a}{v_p} A^* \\ Y_T - Y_P^* - B^* - C^* \\ X_T - X_P^* - A^* - B^* - C^* \end{pmatrix}.$$

where $\gamma = \frac{v_a}{v_k} + \frac{v_a}{v_p}$, $\alpha = 1 + (1+\gamma)\frac{k_1 T}{k_1^{'} + v_a}$, $\beta = \gamma \frac{k_1 T}{k_1^{'} + v_a}$, $\psi = \frac{k_2^{'} + v_k}{\gamma k_2}\frac{v_a}{v_k}$,

$\eta = \frac{\alpha}{\beta}(Y_T - Y_P^{*}) + \psi - X_T$ and $\sigma = -\psi X_T$.

The fixed point *F* exists for every $Y_T \geq 0$. However, F^* exists only for $Y_T \geq Y_P^{*}$, (SI Fig. 4). The point $Y_T = Y_P^{*}$ is a transcritical bifurcation (5) of the system[a].

The steady state output associated with F^*, that is, $Y_P^{*} = \frac{k_3^{'} + v_p}{k_3}\frac{v_a(s)}{v_p}$, is robust to all component concentrations (SI Fig. 4), because none of the concentrations affect the value of this expression. The output is modulated by the input signal *s* through its effect on rate constants. Note that at the fixed point F^*, the ATP consumption rate is positive (Eq. **11**). In contrast, at the fixed point *F*, the ATP consumption rate is zero. The steady state output in this case, $Y_P = Y_T$, is not robust to changes in Y_T and cannot be modulated by the input signal *s*.

Stability of steady state solutions:

We show below that the system tends towards the steady state solution *F* for any choice of positive rate constants and positive initial concentrations, provided that $Y_T < Y_P^{*}$. However, *F* becomes unstable in the region $Y_T > Y_P^{*}$ (SI Fig. 4), and the robust fixed point F^* is locally stable. This can be seen by considering the function

[a] A note regarding the fixed point *F*: in this fixed point, all of X and Y are phosphorylated. If no other reactions are present, once this state is entered the system remains there indefinitely, even when F^* is the only stable fixed point. Thus, this state can be called an "absorbing state". In principle, stochastic fluctuations can cause entry into this state (6). However, in realistic systems, there are several mechanisms that allow "escape" form this state. For example, expression of new X and Y proteins, which occurs on a slow timescale compared to the phosphorylation reactions, as well as spontaneous dephosphorylation of the proteins, ensures that the system does not remain in this state forever.

[12] $L = (k_3' + v_p)(X + Y + A + B) + (k_3' + 2v_p)C$.

Note that $L \geq 0$ for every value of the independent concentrations *X*, *Y*, *A*, *B* and *C* because the concentrations are non-negative and the coefficients are positive. That all concentrations remain non-negative is guaranteed by a basic result in chemical reaction network theory (7).The function *L* is equal to zero if and only if the system is at the fixed point *F*, since *L* is a non-negative, linear function of all five independent concentrations. Using **3**, **5**, **6**, **7**, **9** and **12**:

[13] $$\frac{dL}{dt} = k_3 v_p A(Y_P - Y_P^*).$$

We see by inspecting **13** that in the region $Y_T < Y_P^*$, $\dot{L} < 0$ if $A > 0$, and $\dot{L} = 0$ if $A = 0$. Thus, *L* continues to decrease until *A* reaches zero and stays there. This can happen only if the system is at *F*, since *F* is the only fixed point in the region $Y_T < Y_P^*$. Hence, *F* is the sole attractor of the system in the region $Y_T < Y_P^*$.

F ceases to be stable in the region $Y_T > Y_P^*$. This can be seen by linearizing Eq. **13** around *F*:

[14] $$\frac{d\Delta L}{dt} = k_3 v_p \Delta A(Y_T - Y_P^*).$$

Inspecting Eq. **14** shows that in the region $Y_T > Y_P^*$, $\Delta\dot{L} \geq 0$ (since $\Delta A \geq 0$), and therefore the fixed point *F* is not stable.

The dynamic behavior of the system in the region $Y_T > Y_P^*$ is determined by five independent differential equations and in general depends on the values of all the rate constants and initial conditions. However, a simplification is possible if in each of Eqs. **1'a–1'c** the rates of the binding and dissociation reactions of each reaction pair are much faster than the rates of the respective covalent modification reactions (i.e. phosphorylation / dephoshorylation). Such separation of timescales occurs commonly

in enzymes (e.g. Michaelis-Menten model (8)). This timescale separation condition can be expressed mathematically by the following inequalities:

$$[15] \quad \begin{aligned} v_a &<< k_1', k_1 \frac{XT}{A}, \\ v_k &<< k_2', k_2 \frac{X_P Y}{B}, \\ v_p &<< k_3', k_3 \frac{AY_P}{C}. \end{aligned}$$

Hence, whenever timescale separation exists and conditions **15** are satisfied, the system approaches quasi equilibrium much faster than it approaches steady state, and the following independent quasi equilibrium relations result:

$$[16] \quad A = \frac{k_1}{k_1' + v_a} XT,$$

$$[17] \quad B = \frac{k_2}{k_2' + v_k} X_P Y,$$

$$[18] \quad C = \frac{k_3}{k_3' + v_p} AY_P.$$

Using **1**, **2** and **16–18** in **3–9** results in two differential equations that govern the behavior of the system:

$$[19] \quad \frac{dP}{dt} = v_a A \left(1 - \frac{Y_P}{Y_P^*} \right),$$

$$[20] \quad \frac{dY_P}{dt} = v_k B - v_p C,$$

where $P = X_P + Y_P$, and A, B and C are expressed as functions of P and Y_P.

Using the simplified equations **19** and **20**, as well as the quasi equilibrium[b] conditions **16–18**, it is possible to show that F^* is a locally stable fixed point. This is done by perturbing Eqs. **19** and **20** and linearizing the resulting equations:

$$\frac{d\Delta Y_P}{dt} = -a\Delta Y_P + b\Delta P, \tag{21}$$

$$\frac{d\Delta P}{dt} = -c\Delta Y_P, \tag{22}$$

where

$$\begin{aligned} a &= \frac{v_p(1+a_0+a_2+a_1a_2)+v_k(1+a_1+a_3+a_0a_3)}{a_2a_3-1}, \\ b &= \frac{v_p(a_0+a_2)+v_k(1+a_0a_3)}{a_2a_3-1}, \\ c &= v_a\frac{A^*}{Y_P^*}, \end{aligned} \tag{23}$$

$$\begin{aligned} a_0 &= \frac{Y^*}{X_P{}^*}, \\ a_1 &= \left(1+\frac{\tilde{k}_1}{T}\right)\frac{A^*}{Y_P{}^*}, \\ a_2 &= 1+\frac{\tilde{k}_2}{X_P{}^*}, \\ a_3 &= 1+\left(1+\frac{\tilde{k}_1}{T}\right)\frac{v_p}{v_a}, \end{aligned} \tag{24}$$

and

[b] This is always possible because all the concentrations corresponding to F^* are greater than zero. Thus, there is always a region of concentration space where conditions **15** are satisfied.

$$\tilde{k}_1 = \frac{k_1' + v_a}{k_1},$$

[25] $$\tilde{k}_2 = \frac{k_2' + v_k}{k_2},$$

$$\tilde{k}_3 = \frac{k_3' + v_p}{k_3}.$$

The characteristic equation of **21** and **22** is

[26] $$\lambda^2 + a\lambda + bc = 0,$$

which yields the eigenvalues

[27] $$\lambda_{\pm} = \frac{-a + \sqrt{a^2 - 4bc}}{2}, \quad \frac{-a - \sqrt{a^2 - 4bc}}{2}.$$

Inspecting **23**–**25** shows that *a*, *b* and *c* are always positive, and therefore the eigenvalues **27** always have negative real parts. Thus, the fixed point F^* is stable when timescale separation conditions **15** hold, and F^* is the only attractor of the system in the region $Y_T > Y_P^*$. We note that the separation of timescales is merely an assumption made for simplicity in the analysis of stability. Below, we show that this assumption is not needed for stability, as demonstrated by numerical analysis of the equations with general rate constants.

Numerical analysis of the system:

We studied the general behavior of the system using numerical analysis (Matlab 7.04, The MathWorks), even without timescale separation. The main result is the same as in the case where timescale separation applies: the system always approaches *F* if $Y_T < Y_P^*$, whereas the system always approaches F^* if $Y_T > Y_P^*$.

We explored the possible steady states of the system by systematically changing Y_T and then numerically solving the temporal dynamics of the system, while keeping all

rate constants fixed. We found that in agreement with the analytic result, as long as $Y_T > Y_P^*$, the output of the system settles into the robust steady state Y_P^* (SI Fig. 4). For $Y_T \leq Y_P^*$ the system settles into a steady state where all the response regulator proteins are phosphorylated: $Y_P^* = Y_T$ (SI Fig. 4).

Next, we analyzed the behavior of the system for a wide range of rate constant values. To do this, we used a Monte Carlo approach in which each rate constant and initial condition was (log) randomly and independently chosen in the range [1, 100]. We simulated the system's behavior 1000 times and found that in all cases the system settled into a stable steady state as predicted by the theoretical analysis ($<10^{-3}$ relative difference) with no cases of oscillatory limit cycles or chaotic behavior.

Finally, we followed the response of the model to a step-like signal, which causes a modulation of the autophosphorylation rate (SI Fig. 5). We found that a change in v_a results in a robust change in the system's output: Systems equal in all aspects except protein or ATP concentrations reach the same steady state output following a modulation of v_a. However, the timescale of approach to steady state does depend on the protein and ATP concentrations. It is interesting to note that for physiological parameters the response time is minimal at a defined value of X_T (SI Fig. 6). Response time on the order of minutes can be reached with tens to hundreds of copies of the sensor, in accord with measured concentrations of sensors such as EnvZ (12).

The effect of additional reactions on input-output robustness:

The analysis presented above demonstrates that mechanism **1'** has input-output robustness. We now study how this property may be affected by additional possible reactions involving the components in **1'**. The main result is that when the flux through an additional reaction is of order ε compared to the corresponding reaction in the original model, robustness is affected only to order ε. Thus, robustness can be made as close to perfect as desired by appropriate choice of rate constants for the additional reactions.

We considered the effect of seven such possible reactions, all of which might plausibly exist in signaling systems of the present type: (a) autodephosphorylation of the sensor, $X_P \xrightarrow{\alpha_X} X + P_i$; (b) autodephosphorylation of the response regulator: $Y_P \xrightarrow{\alpha_Y} Y + P_i$; (c) reverse autophosphorylation: $X_P + D \xrightarrow{v'_a} A$; (d) reverse phosphotransfer: $X + Y_P \xrightarrow{v'_k} B$; (e) reverse dephoshorylation: $A + Y + P_i \xrightarrow{v'_p} C$; (f) Use of ADP as a co-factor for response regulator dephoshorylation in addition to ATP: $X + D \underset{k'_6}{\overset{k_6}{\rightleftarrows}} F$, $Y_P + F \underset{k'_7}{\overset{k_7}{\rightleftarrows}} G \xrightarrow{v_d} F + Y + P_i$, where F denotes the complex X·D and G denotes the complex $X{\cdot}D{\cdot}Y_P$; and (g) an additional pathway for the formation of the ternary complex C: $X + Y_P \underset{k'_4}{\overset{k_4}{\rightleftarrows}} E$, $E + T \underset{k'_5}{\overset{k_5}{\rightleftarrows}} C$. To study the effect of each of the additional reactions on input-output robustness, we added each of a–g separately to the reactions of mechanism **1'**, derived the induced differential equations and analyzed the solutions.

In the case of the reverse phosphotransfer reaction (d), one can show analytically (*SI Appendix 1*) that at steady state, $Y_P = Y_P^*$. Thus, the presence of the reverse phosphotransfer reaction (d) has no influence on input – output robustness, and the conditions for input-output robustness are the same as in the unperturbed mechanism **1'**.

In the case of ADP-catalyzed response regulator dephosphorylation (f), one can derive (*SI Appendix 2*) an analytic expression for Y_P:

[28] $$Y_P = \frac{Y_P^*}{1 + \kappa \frac{D}{T}},$$

where

[29] $$\kappa = \frac{v_d}{v_p} \frac{k'_1 + v_a}{k_1} \frac{k'_3 + v_p}{k_3} \frac{k_7}{k'_7 + v_d} \frac{k_6}{k'_6}.$$

Eq. **28** shows that robustness is maintained with respect to all component concentrations, except the ADP to ATP ratio *D/T*. However, this sensitivity to *D/T* is very small, fundamentally because the *D/T* ratio is very small in cells (D/T~0.05 (9)). Thus, even if ADP is as effective as ATP as a cofactor, its low concentration relative to ATP makes this reaction have a very small effect on robustness. To explicitly calculate the departure from robustness, one can compute the sensitivity χ of the system, defined as the percentage change in Y_P given 1% change in *D/T*, by taking the logarithmic derivative of Y_P with respect to *D/T*: $\chi = \partial \log Y_P / \partial \log(D/T)$. Evaluating the logarithmic derivative using **28** yields

$$[30] \qquad \chi = -\frac{\kappa \frac{D}{T}}{1+\kappa \frac{D}{T}}.$$

Under physiological conditions (parameters as in SI Fig. 5) supplemented by $k_6 = k_7 =$ 100 [sec μM]$^{-1}$ (diffusion limit), $k_6' = 3\cdot10^4$ sec^{-1} (10), $k_7' = 2\cdot10^2$ sec^{-1} (same as k_3', which reflects a similar affinity for Y_P to both A and F) and $v_d = 1$ sec^{-1} (same as v_p, which reflects similar efficiency for ADP and ATP as dephoshorylation coenzymes), one obtains $\kappa = 0.2$. Since in wild-type *E. coli D/T* ~0.05 (9), it follows from **30** that the sensitivity in the present case is approximately $\chi \sim -0.01$. Thus, a 1% change in *D/T* produces approximately -0.01% change in the output Y_P. This predicts that under physiological conditions the system is highly insensitive to the ADP-catalyzed dephoshorylation reaction, thereby preserving input-output robustness to an excellent approximation.

The effects of the sensor autodephosphorylation reaction (a), the response regulator autodephosphorylation reaction (b), the reverse autophosphorylation reaction (c) and the reverse dephoshorylation reaction (e) were studied using numerical analysis (Matlab 7.04, The MathWorks). For each of these additional reactions, we analytically derived conditions on the parameters that tend to make the phosphoryl flux through these reactions small compared to the flux through the corresponding reactions of mechanism **1'** (*SI Appendix 3*). The relative magnitude of these fluxes

was characterized by a dimensionless parameter ε. The goal was to see how input-output robustness varies as a function of ε. A deviation that approaches zero as ε tends to zero signifies stability of the robust input-output mechanism **1'** under model structural uncertainty.

For each of the reactions (a–c and e), we randomly chose each independent rate constant from the interval whose limits are 0.5 the wild-type level to 5 times the wild-type level (wild-type values are as in SI Fig. 5 and as in the preceding paragraph). This was repeated 100 times. For each such choice of rate constants we considered five values of ε in the range [10^{-3} 10^{-1}]. For each of the 100 choices of independent rate constants and five choices of ε, we randomly chose 500 values of X_T, Y_T, T, D (when applicable) and P_i (when applicable). The interval whose limits are 0.5 the wild-type level to 5 times the wild-type level was used for all total concentrations except Y_T, where the interval $[2Y_P^* \; 20Y_P^*]$ was used, so that $Y_P^* < Y_T$. For each choice of independent rate constants, ε and initial concentrations we calculated the remaining dependent rate constants using the relevant "smallness" condition (*SI Appendix 3*), randomly generated the initial concentrations corresponding to the total concentrations chosen, integrated the differential equations over 10^9 msec (to ensure sufficient time for relaxation to steady state) and obtained the terminal value of the output Y_P. Finally, for each choice of independent rate constants and ε we calculated the coefficient of variation of the output due to variation in component concentrations (defined as the standard deviation of Y_P divided by the mean of Y_P of the 500 terminal values of Y_P). The coefficient of variation is a measure of the effect of each additional reaction on input-output robustness. The smaller it is, the closer the system is to having perfect input-output robustness.

The results of the simulations appear in Table 2. We find that for each of the 100 random choices of independent rate constants, the coefficient of variation C_v is, to an excellent approximation, linear in $\varepsilon : C_v = m\varepsilon$. Hence, insofar as parameters are chosen such that each of the additional reactions is "small" compared to the corresponding reactions of the mechanism **1'**, their effect on input-output robustness is of the same order of smallness.

To study the effect of the additional pathway (g) for forming the ternary complex C, we performed numerical simulations similar to those described in the previous paragraph. We hypothesized that if ATP saturates X whereas Y_P does not saturate X, that is if $\varepsilon = \frac{(k'_1 + v_a)/k_1}{T}$ is small compared to $\frac{(k'_2 + v_k)/k_2}{Y_P^*}$, the primary pathway for the formation of the ternary complex would be that of mechanism **1'** rather than that of additional reactions (g). Indeed, for all 100 random choices of independent rate constants and for all $\varepsilon < 0.32$ in the range [0.01 1], the coefficient of variation of the output Y_P was less than 10^{-4}. In typical wild-type parameters, one finds $\varepsilon = 0.06$. We thus conclude that under typical parameters (and a neighborhood thereof) the effect of the putative additional pathway on input-output robustness is very small.

In conclusion, conditions can be found, for all seven additional reactions considered, such that the deviation from input-output robustness of mechanism **1'** is very small. The wild-type parameters seem to assure a small effect on input-output robustness in the case of ADP-catalyzed response regulator dephoshorylation (by the small values of D/T and κ), additional pathway for the formation of the ternary complex C (by the high affinity of ATP to free sensor) and response regulator autodephosphorylation (by the long half-life of the phosphoryl modification of the response regulator species, Table 1). None of the additional reactions modified the dynamic behavior observed for mechanism **1'** in the range of parameters tested. The evidence presented supports the notion that, in an appropriate range of parameter values, mechanism **1'** is stable against model structural uncertainty described by reactions a to g above, and that wild-type parameter values, where known, appear to be in this "robust" range.

The linear model and integral feedback:

In this section, we relate the present model (in a linearized version) to the control theory principle of integral feedback. Integral feedback helps explain the stability of the output value, but additional features of the mechanism ensure that this output value is robust to all component concentrations. Consider the linearized model described above, Eqs. **21** and **22**. Define a new variable $\Delta Q = -\Delta P / c$, and substitute into equations **21** and **22**. The result is

[31] $$\frac{d\Delta Y_P}{dt} = -a\Delta Y_P - bc\Delta Q\,,$$

[32] $$\frac{d\Delta Q}{dt} = \Delta Y_P.$$

Eq. **32** states that ΔQ is the time integral of the deviation ΔY_P of the output from its steady state value. Eq. **31** shows that ΔQ is negatively fed back to determine the rate of change of the deviation ΔY_P. This shows that Eqs. **31** and **32** contain integral feedback control (1).

Yi et al have shown that integral control is required for perfect adaptation, as observed in bacterial chemotaxis (1). In that system, the steady state value of the output depends on the concentrations of some of the system components (the proteins CheR and CheB), and is independent of the external signal (attractant concentration). Integral feedback ensures that this steady state output is asymptotically tracked at all input levels, since the steady state output does not depend on the attractant (input) level. In contrast, the steady state output of the present model is independent of all total component concentrations in the system, yet is responsive to the level of the input signal (osmolarity) through signal-sensitive rate constants. As in chemotaxis, integral feedback ensures that the steady state output of the present model is asymptotically tracked. This is the source of the stability observed in the present model. However, integral feedback is not sufficient to guarantee the independence of the steady state output from all total component concentrations, as the chemotaxis example shows. Thus, input-output robustness is a feature of the present model that does not follow from integral feedback alone.

Remarks on a previous model of the EnvZ/OmpR system:

A model of the EnvZ/OmpR system has been proposed by Batchelor and Goulian (3), along with their experimental study of robustness in this system. Here we note that in this model the output is proportional to ATP levels, $Y_P \sim ATP$. In addition, robustness with respect to protein levels in this model requires that $Y_P << Y_T$.

Equation **1** of the model of ref. 3 gives the output Y_P as a function of Y_T, ATP and various rate constants:

[33] $$Y_P = \frac{1}{2}(C_t + C_p + Y_T) - \frac{1}{2}\sqrt{(C_t + C_p + Y_T)^2 - 4C_p Y_T} + \ldots$$

The ATP dependence enters through C_p, which is linear in ATP: $C_p \sim ATP$ (3). C_t is a function of rate constants only.

In the limit $C_t + C_p << Y_T$, it is possible to Taylor-expand the right-hand side of Eq. **33** in the parameter $\frac{C_t + C_p}{Y_T}$. Neglecting all terms that are first-order or higher in this parameter one arrives at Eq. **2** of ref. 3:

[34] $$Y_P = C_p + \ldots$$

This shows that in the limit $C_t + C_p << Y_T$, which according to Eq. **34** corresponds to $Y_P << Y_T$, the dependence of Y_P on Y_T is lost. However, since $C_p \sim ATP$ one has

[35] $$Y_P \sim ATP.$$

Thus the linear dependence of Y_P on ATP is maintained for all values of Y_T for which Eq. **34** is valid. This ATP-dependence cannot be alleviated even if the sensor is saturated with ATP. The intuitive reason is that the sensor-ATP complex causes phosphorylation, but sensor alone causes dephoshorylation, and hence ATP is not cancelled out between these two opposing reactions.

More generally, the main text proposes a "principle" for generation of robust input-output relations, in which the influx of phosphoryl depends on all components (excluding Y_P) in the same functional way as the outflux. The model of ref. 3 does not obey this condition since influx depends on ATP but outflux does not.

In summary, in the model of ref. 3 the output Y_P generally depends on both Y_T and ATP. In the limit where only a small fraction of response regulator is phosphorylated, $Y_P << Y_T$, the output becomes robust with respect to Y_T and remains sensitive to variations in ATP levels.

Appendix 1: Steady state output in the presence of reverse phosphotransfer:

The chemical reactions in the system are:

$$\text{(a)}\quad \mathrm{X} + \mathrm{T} \underset{k'_1}{\overset{k_1}{\rightleftharpoons}} \mathrm{A} \xrightarrow{v_a(s)} \mathrm{X_P} + \mathrm{D},$$

[35] $$\text{(b)}\quad \mathrm{X_P} + \mathrm{Y} \underset{k'_2}{\overset{k_2}{\rightleftharpoons}} \mathrm{B} \underset{v'_k}{\overset{v_k}{\rightleftharpoons}} \mathrm{X} + \mathrm{Y_P},$$

$$\text{(c)}\quad \mathrm{A} + \mathrm{Y_P} \underset{k'_3}{\overset{k_3}{\rightleftharpoons}} \mathrm{C} \xrightarrow{v_p} \mathrm{A} + \mathrm{Y} + \mathrm{P_i}.$$

This deviation from the mechanism **1'** does not modify Eqs. **1-7** in the main text. Hence, by Eq. **7** of the main text, $Y_P = Y_P^*$.

Appendix 2: Steady state output in the presence of ADP-catalyzed dephoshorylation of the response regulator:

The chemical reactions in the system are:

$$\text{(a)}\quad \mathrm{X} + \mathrm{T} \underset{k'_1}{\overset{k_1}{\rightleftharpoons}} \mathrm{A} \xrightarrow{v_a(s)} \mathrm{X_P} + \mathrm{D},$$

$$\text{(b)}\quad \mathrm{X_P} + \mathrm{Y} \underset{k'_2}{\overset{k_2}{\rightleftharpoons}} \mathrm{B} \xrightarrow{v_k} \mathrm{X} + \mathrm{Y_P},$$

[36] $$\text{(c)}\quad \mathrm{A} + \mathrm{Y_P} \underset{k'_3}{\overset{k_3}{\rightleftharpoons}} \mathrm{C} \xrightarrow{v_p} \mathrm{A} + \mathrm{Y} + \mathrm{P_i},$$

$$\text{(d)}\quad \mathrm{X} + \mathrm{D} \underset{k'_6}{\overset{k_6}{\rightleftharpoons}} \mathrm{F},$$

$$\text{(e)}\quad \mathrm{Y_P} + \mathrm{F} \underset{k'_7}{\overset{k_7}{\rightleftharpoons}} \mathrm{G} \xrightarrow{v_d} \mathrm{F} + \mathrm{Y} + \mathrm{P_i}$$

Inspection of **36** shows that phosphoryl groups may become covalently bound to protein components of the system only through the autophosphorylation reaction **36 (a)**. However, phosphoryl groups may be removed from the system by either of the two dephoshorylation routes **36 (c)** or **36 (e)**. Thus, the phosphoryl influx and outflux are

[37] $J_i = v_a A,$

[38] $J_o = v_p C + v_d G.$

Inspection of reactions **36** shows that Eqs. **3** and **9** are valid also in the present case. Using **9**, **37**, **38** and the steady state conditions $J_i = J_o$ and $\dot{C} = 0$ yields

[39] $$Y_P = Y_P^*\left(1 - \frac{v_d}{v_a}\frac{G}{A}\right).$$

Using **3** and **9** in steady state yields Eq. **16**. The differential equations for *F* and *G* are:

[40] $$\dot{F} = k_6 XD - k'_6 F + (k'_7 + v_d)G - k_7 F Y_P,$$

[41] $$\dot{G} = k_7 F Y_P - (k'_7 + v_d)G.$$

Eq. **41** at steady–state yields:

[42] $$G = \frac{k_7}{k'_7 + v_d} F Y_P.$$

Using **40** and **41** at steady state yields:

[43] $$F = \frac{k_6}{k'_6} XD.$$

Using **16**,**42** and **43** in **39**, and rearranging terms yields the required result, Eq. **28**.

Appendix 3: Conditions for making fluxes of added reactions small compared to primary model reactions:

Here we derive conditions on the parameters of the additional reactions that tend to make their fluxes small in comparison with the fluxes of the relevant reactions of the main model. We first consider the case of sensor autodephosphorylation: $\mathrm{X_P} \xrightarrow{\alpha_X} \mathrm{X} + \mathrm{P_i}$. The phosphoryl outflux through this reaction is $J'_o = \alpha_X X_P$. We wish to find conditions that ensure that this rate is small compared to the overall phosphoryl outflux J_o. When this is the case, J_o is well-approximated by the phosphoryl outflux of mechanism **1'**: $J_o \approx v_p C = v_k B$. Using the steady state version of Eq. **6**, which is also valid in the present case, yields $B = [k_2/(k'_2 + v_k)] X_P Y$. Requiring that $J'_o << J_o$ implies that $\alpha_X X_P << v_k [k_2/(k'_2 + v_k)] X_P Y$. Eliminating X_P from both sides yields :

$$[44] \qquad \alpha_X << v_k \frac{k_2}{k'_2 + v_k} Y.$$

Assuming that $Y_T >> X_T$ (as in the wild-type case, SI Fig. 5), $Y \approx Y_T - Y_P^*$. Since in the simulations we choose Y_T from the range $[2Y_P^* \; 20Y_P^*]$, it follows that $Y > Y_P^*$. Hence, requiring that

$$[45] \qquad \alpha_X = \varepsilon v_k \frac{k_2}{k'_2 + v_k} Y_P^*,$$

where $\varepsilon << 1$, ensures that **44** is fulfilled, and therefore implies $J'_o << J_o$. Thus, Eq. **45** is the smallness condition in the present case.

We next consider the case of response regulator autodephosphorylation: $\mathrm{Y_P} \xrightarrow{\alpha_Y} \mathrm{Y} + \mathrm{P_i}$. The phosphoryl outflux through this reaction is $J'_o = \alpha_Y Y_P$. We wish to find conditions that ensure that this rate is small compared to the overall phosphoryl outflux J_o. When this is the case, J_o is well-approximated by the

phosphoryl outflux of mechanism **1'**: $J_o \approx v_p C = v_a A$. One also expects that $A \approx A^*$ and $Y_P \approx Y_P^*$. This implies that $J_o^{'} \approx \alpha_Y Y_P^*$ and $J_o \approx v_a A^*$. By Eq. **11**, $A^* = \frac{\beta}{\alpha\gamma}(X_T - X_P{}^*)$, where α,β and γ are as in P. 4 of this supporting information document. Thus, $J_o \approx v_a \frac{\beta}{\alpha\gamma}(X_T - X_P{}^*)$. Requiring that $J'_o << J_o$ implies that $\alpha_Y << v_a \frac{\beta}{\alpha\gamma}\frac{X_T - X_P{}^*}{Y_P^*}$, and therefore the condition

[46] $\alpha_Y = \varepsilon v_a \frac{\beta}{\alpha\gamma}\frac{X_T}{Y_P^*}$,

where $\varepsilon << 1$, insures that $J'_o << J_o$ is fulfilled (when $X_T - X_P{}^* \sim X_T$). Hence, Eq. **46** is the smallness condition in the present case.

The smallness condition in the case of the reverse autophosphorylation reaction $\mathrm{X_P + D \xrightarrow{v'_a} A}$ follows easily from the case of sensor autodephosphorylation presented above. Since $J_o^{'} = v'_a DX_P$ in the former case and $J_o^{'} = \alpha_X X_P$ in the latter, since D is constant and since the rationale of the derivation of the smallness conditions in both cases is virtually identical, it is only required to substitute $v'_a D$ for α_X in **45** to obtain:

[47] $v'_a = \varepsilon \frac{v_k}{D}\frac{k_2}{k'_2 + v_k} Y_P^*$.

Finally, the smallness condition in the case of reverse dephoshorylation, $\mathrm{A + Y + P_i \xrightarrow{v'_p} C}$, is obtained as follows. The phosphoryl influx through this reaction is $J'_i = v'_p AYP_i$. Assuming that this is small compared to the total phosphoryl influx J_i, one can approximate the rate of phosphoryl gain by the autophosphorylation reaction of **1'** using $J_i \approx v_a A = v_p C$. Moreover, $A \approx A^*$ and $C \approx C^* = \frac{k_3}{k'_3 + v_p} A^* Y_P^*$, which follows from Eq. **9** applied at steady state. Thus, the

requirement $J'_i << J_i$ implies that $v'_p A^* YP_i << v_p \frac{k_3}{k'_3+v_p} A^* Y_P^*$. Eliminating A^* from both sides yields:

$$[48] \qquad v'_p << v_p \frac{k_3}{k'_3+v_p} \frac{Y_P^*}{YP_i}.$$

Since in the simulations we choose Y_T from the range $[2Y_P^* \; 20Y_P^*]$, it follows that $Y_P^* / Y > 1/19$. Thus, by requiring that

$$[49] \qquad v'_p = \varepsilon \frac{v_p}{19P_i} \frac{k_3}{k'_3+v_p},$$

we ensure that **48** is satisfied, and $J'_i << J_i$ is fulfilled.

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Table 2. Effects of additional reactions on input-output robustness

Additional reaction	Smallness condition	$C_v = m\varepsilon$			
		m(wild-type)	<m>	σ_m	Range of m
(a) auto-dephosphorylation of the sensor: $X_P \xrightarrow{\alpha_X} X + P_i$	$\alpha_X = \varepsilon v_k \frac{k_2}{k'_2 + v_k} Y_P^*$	0.18	0.16	0.014	[0.12 0.20]
(b) auto-dephosphorylation of the response regulator: $Y_P \xrightarrow{\alpha_Y} Y + P_i$	$\alpha_Y = \varepsilon \frac{\beta}{\alpha\gamma} \frac{X_T}{Y_P^*}$	0.33	0.14	0.38	[0.29 1.41]
(c) reverse autophosphorylation: $X_P + D \xrightarrow{v'_a} A$	$v'_a = \varepsilon \frac{v_k}{D} \frac{k_2}{k'_2 + v_k} Y_P^*$	0.45	0.42	0.036	[0.34 0.51]
(e) reverse dephoshorylation: $A + Y + P_i \xrightarrow{v'_p} C$	$v'_p = \varepsilon \frac{v_p}{19 P_i} \frac{k_3}{k'_3 + v_p}$,	0.92	0.90	0.030	[0.83 0.96]

The smallness conditions, which ensure that the additional reactions are of order ε relative to the relevant reactions of mechanism **1'**, are derived in *SI Appendix 3*. Robustness is quantified by means of the coefficient of variation C_v of the output for random choices of the component concentrations. This turns out to be linear with epsilon: $C_v = m\varepsilon$ in the range $\varepsilon \in [10^{-3}\ 10^{-1}]$ for reactions a, b, c and e. m(wild-type) denotes the slope m of $C_v = m\varepsilon$ for the wild-type values of the rate constants and initial concentrations (SI Fig. 5 supplemented by D = 53 μM (9, 11), P_i = 1000 μM). <m> and σ_m are the mean and standard deviation, respectively, of the slope m, evaluated over 100 random choices of independent rate constant values. The range of m gives the minimum and maximum value of m over all 100 random choices of rate constants. Reactions d,f, and g are treated in the SI text.

Fig. 4. Steady state output Y_P as a function of the total response regulator concentration Y_T. Two steady state outputs exist in the region $Y_T > Y_P^*$: $Y_P = Y_T$ (unstable, dashed line) and $Y_P = Y_P^*$ (stable, solid line). Only one steady state solution exists in the region $Y_T < Y_P^*$: $Y_P = Y_T$ (stable, solid line). The stable steady state solution was obtained by numerically integrating Eqs. **3-9** with parameters $k_1 = k_2 = k_3 = 1$ μM^{-1} sec^{-1}, $k'_1 = k'_2 = k'_3 = v_a = v_k = v_p = 1$ sec^{-1}, $T = 1$ μM, $X_T = Y_T = 10$ μM. In this example $Y_P^* = 2$ μM. Blue lines: phase trajectories corresponding to initial conditions where Y_P values are below the stable steady state value. All blue lines converge to the stable steady state. Red lines: phase trajectories corresponding to initial conditions where the initial values of Y_P are above the stable steady state value. All red lines also converge to the stable steady state.

Fig. 5. Dynamics of the output Y_P in response to a step-like increase in input signal. Blue line: wild-type system modeled after the EnvZ/OmpR system of *E. coli*. Parameters are $Y_T = 3.5$ μM (12), $X_T = 0.1$ μM (12), $T = 1000$ μM (13), diffusion limited $k_1 = k_2 = k_3 = 100$ μM^{-1}sec^{-1}, $k'_1 = 6 \cdot 10^3$ sec^{-1} (10), $k'_2 = 200$ sec^{-1} (14). $k'_3 = 200$ sec^{-1}, $v_k = v_p = 1$ sec^{-1}, $v_a = 0.2$ sec^{-1} and 1.0 sec^{-1} before and after step-like increase, respectively. Red line: same as the wild-type system, except that X_T is tenfold higher. Light blue line: same as the wild-type system, except that *ATP* is tenfold lower. Green line: same as wild-type system, except that Y_T is tenfold higher. Note that steady state levels are independent of protein and ATP concentrations. Response time is dependent on these parameters. Similar dynamics are observed as long as $Y_T > Y_P^*$.

Fig. 6. Response time as a function of total sensor concentration X_T. Parameters other than X_T are as in the wild-type system of SI Fig. 5. The response time decreases like $1/X_T$ for small X_T.