Molecular titration promotes oscillations and bistability in minimal network models with monomeric regulators

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Supplementary Information

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Contents

1 Analysis of the inhibited and activated modules

The oscillator and the bistable systems considered in the main paper can be analyzed following the theory proposed in [1, 2], which allows us to show that they structurally have (by design) the capacity to respectively exhibit oscillations and bistability.

On the one hand, it can be shown that a system given by the feedback interconnection of an inhibitor module and an activator module is the negative feedback interconnection of two monotone systems, and is therefore a strong candidate oscillator according to [2] (if driven to instability, the system necessarily exhibits sustained oscillations). On the other hand, a system given by the feedback interconnection of mutually inhibiting modules is the positive feedback interconnection of two monotone systems, and is therefore a strong candidate bistable system according to [2] (if the system is driven to instability, bistable phenomena necessarily arise).

Here we demonstrate the same properties following an alternative, simplified route. We begin by analyzing the properties of the inhibited and the activated modules individually.

1.1 Analysis of the inhibited module

1.1.1 Analysis in the absence of direct titration reaction

We recall the model for the inhibited module:

$$\begin{aligned}
\dot{x}_T &= \alpha(x_T^{tot} - x_T)x_A - \delta x_T r_I && (1)\\
\dot{x}_A &= \kappa(x_A^{tot} - x_A - x_T) - \alpha(x_T^{tot} - x_T)x_A && (2)\\
\dot{r}_I &= \beta u_I - \delta x_T r_I - \phi r_I. && (3)
\end{aligned}$$

Assumption 1. *We assume that $x_A^{tot} \geq x_T^{tot}$.*

Additionally, we make an assumption that relates parameters κ, α, and the total concentrations of target and constitutive activator.

Assumption 2. *We assume that $x_A^{tot} - x_T^{tot} - \frac{\kappa}{\alpha} \geq 0$.*

Proposition 1. *For a given concentration of inhibitor source $\bar{u}_I$, system (1)–(3) has unique equilibrium values $\bar{x}_T$, $\bar{x}_A$ and $\bar{r}_I$. The equilibrium $\bar{x}_T$ is a monotonic, strictly decreasing function of $\bar{u}_I$.*

Proof. We first find an expression of $\bar{x}_A$ as a function of $\bar{x}_T$. From $\dot{x}_A = 0$, we obtain:

$$\bar{x}_A(\bar{x}_T) = \frac{\kappa(x_A^{tot} - \bar{x}_T)}{\alpha(x_T^{tot} - \bar{x}_T) + \kappa} \doteq g_A(\bar{x}_T).$$

It can be verified that under Assumptions 1 and 2, $\partial g_A(\bar{x}_T)/\partial \bar{x}_T \geq 0$. Thus $g_A(\bar{x}_T)$ is a continuous, monotonically increasing function of $\bar{x}_T$, and, for a given value of u_I, $\bar{x}_T$, there is a unique equilibrium $\bar{x}_A$.

Now we find $\bar{r}_I$ as a function of $\bar{x}_T$. From $\dot{x}_T = 0$, We obtain:

$$\begin{aligned}
\bar{r}_I(\bar{x}_T) &= \frac{\alpha(x_T^{tot} - \bar{x}_T)\bar{x}_A(\bar{x}_T)}{\delta \bar{x}_T} = \frac{\alpha(x_T^{tot} - \bar{x}_T)}{\delta \bar{x}_T}\frac{\kappa(x_A^{tot} - \bar{x}_T)}{\alpha(x_T^{tot} - \bar{x}_T) + \kappa}\\
&= \frac{\kappa}{\delta}\frac{(x_A^{tot} - \bar{x}_T)}{\bar{x}_T}\frac{(x_T^{tot} - \bar{x}_T)}{(x_T^{tot} - \bar{x}_T) + \frac{\kappa}{\alpha}} \doteq \frac{\kappa}{\delta} A(\bar{x}_T)\, B(\bar{x}_T),
\end{aligned}$$

where $A(\bar{x}_T) \doteq \frac{(x_A^{tot} - \bar{x}_T)}{\bar{x}_T}$ and $B(\bar{x}_T) \doteq \frac{(x_T^{tot} - \bar{x}_T)}{(x_T^{tot} - \bar{x}_T) + \frac{\kappa}{\alpha}}$. Since $\partial A/\partial \bar{x}_T = -\frac{x_A^{tot}}{\bar{x}_T^2} < 0$, $\partial B/\partial \bar{x}_T = -\frac{\frac{\kappa}{\alpha}}{(x_T^{tot} - \bar{x}_T + \frac{\kappa}{\alpha})^2} < 0$, and both $A(\bar{x}_T)$ and $B(\bar{x}_T)$ are positive for arbitrary parameter values (except at $\bar{x}_T = x_T^{tot}$, where $B(\bar{x}_T) = 0$), we can conclude that the partial derivative $\partial \bar{r}_I(\bar{x}_T)/\partial \bar{x}_T = \frac{\kappa}{\delta}\left[(\partial A/\partial \bar{x}_T)\, B(\bar{x}_T) + A(\bar{x}_T)(\partial B/\partial \bar{x}_T)\right] < 0$, thus $\bar{r}_I$ is a monotonic, strictly decreasing function of $\bar{x}_T$.

Finally, from $\dot{r}_I = 0$ we find

$$\bar{u}_I = \frac{1}{\beta}(\delta \bar{x}_T + \phi)\bar{r}_I(\bar{x}_T) \doteq h(\bar{x}_T).$$

To verify that the introduced function $h(\bar{x}_T)$ is a strictly decreasing monotonic function of $\bar{x}_T$, we rewrite it as $h(\bar{x}_T) = \frac{1}{\beta}\left[\delta \bar{x}_T \bar{r}_I(\bar{x}_T) + \phi \bar{r}_I(\bar{x}_T)\right] = \frac{1}{\beta}\left[C(\bar{x}_T) + \phi r_I(\bar{x}_T)\right]$. Because β and ϕ are positive constants, and we already verified that $\partial \bar{r}_I(\bar{x}_T)/\partial \bar{x}_T < 0$, we only need to check that $\partial C(\bar{x}_T)/\partial \bar{x}_T < 0$. It is sufficient to note that $C(\bar{x}_T) = \kappa(x_A^{tot} - \bar{x}_T)B(\bar{x}_T)$ (see definition of $B(\bar{x}_T)$ above). Since $(\partial B/\partial \bar{x}_T) < 0$, $\partial(x_A^{tot} - \bar{x}_T)/\partial \bar{x}_T < 0$, and both $B(\bar{x}_T) > 0$ and $(x_A^{tot} - \bar{x}_T) > 0$, we have that $\partial C(\bar{x}_T)/\partial \bar{x}_T < 0$.

Being $h(\bar{x}_T)$ a continuous, monotonic, strictly deceasing function of $\bar{x}_T$, its inverse is also a continuous, monotonic, decreasing function: $\bar{x}_T = g(\bar{u}_I) = h^{-1}(\bar{u}_I)$. We conclude that the equilibrium $\bar{x}_T$ for a given $\bar{u}_I$ is unique, and so are the other equilibria $\bar{x}_A$ and $\bar{r}_I$. In particular, the higher the concentration of input U_I, the smaller the equilibrium concentration of X_T. □

We can show that a suitable set is positively invariant for the system: namely, any trajectory starting in this set is confined in the set for all time instants.

Proposition 2. *The set*

$$0 \le x_T \le x_T^{tot}, \qquad \frac{\kappa}{\alpha} \le x_A \le x_A^{tot}, \qquad r_I \ge 0 \tag{4}$$

is positively invariant [3] for the system (1)–(3) *for any* u_I.

Proof. Since all the variables are non–negative and the variables x_T and x_A cannot exceed their total values, all the constraints are obvious with the exception of $x_A \ge \kappa/\alpha$. We show that this constraint cannot be violated: if we start with $x_A(0) \ge \kappa/\alpha$, then the constraint is satisfied for all $t > 0$. In fact, for $x_A = \kappa/\alpha$ we have

$$\dot{x}_A = \kappa(x_A^{tot} - \kappa/\alpha - x_T) - \alpha(x_T^{tot} - x_T)(\kappa/\alpha) = \kappa(x_A^{tot} - x_T^{tot} - \kappa/\alpha) \ge 0$$

due to Assumption 2. □

Proposition 3. *The solutions of system* (1)–(3) *are bounded.*

Proof. Species x_T and x_A are bounded by assumption. The dynamics of the regulator satisfies the inequality $\dot{r}_I(t) \le \beta u_I^{max} - \phi r_I$. By applying the comparison principle [6], we conclude that

$$r_I(t) \le r_I(0)e^{-\phi t} + \beta u_I^{max}(1 - e^{-\phi t})/\phi,$$

which ensures $r_I(t) \le \max\{r_I(0), \beta u_I^{max}/\phi\}$ at any point in time. □

Proposition 4. *The unique equilibrium of system* (1)–(3) *is locally stable.*

Proof. For a given input u_I, the system admits a unique equilibrium (see Proposition 1). The Jacobian matrix

$$J_I = \begin{bmatrix} -(\alpha \bar{x}_A + \delta \bar{r}_I) & \alpha(x_T^{tot} - \bar{x}_T) & -\delta \bar{x}_T \\ -\kappa + \alpha \bar{x}_A & -[\kappa + \alpha(x_T^{tot} - \bar{x}_T)] & 0 \\ -\delta \bar{r}_I & 0 & -(\delta \bar{x}_T + \phi) \end{bmatrix}$$

can be recast as a Metzler matrix (namely, a matrix whose non–diagonal entries are nonnegative) by changing the sign to the last row and column; in fact term $\alpha \bar{x}_A - \kappa > 0$ in view of Proposition 2. A Metzler matrix has exclusively eigenvalues with negative real part (hence, is stable) if and only if all the coefficients of its characteristic polynomial $\det(\lambda I - J_I)$ are positive. A computation of the characteristic polynomial of J_I shows that all its coefficients are positive. □

Remark 1. *Systems whose Jacobian is (or is similar, up to a change of sign, to) a Metzler matrix are called monotone. As we will show later, in the absence of titration both the oscillator and the bistable system can be seen as the interconnection of two monotone components (corresponding to the modules).*

1.1.2 Analysis in the presence of direct titration reactions

When titration reactions are present, the equations describing the system become:

$$\begin{aligned} \dot{x}_T &= \alpha(x_T^{tot} - x_T)x_A - \delta x_T r_I & (5)\\ \dot{x}_A &= \kappa(x_A^{tot} - x_A - x_T) - \alpha(x_T^{tot} - x_T)x_A - \nu x_A r_I & (6)\\ \dot{r}_I &= \beta u_I - \delta x_T r_I - \phi r_I - \nu x_A r_I. & (7) \end{aligned}$$

Proposition 5. *The solutions of system* (5)–(7) *are globally bounded.*

Proof. All of the variables are non–negative and the variables x_T and x_A are upper–bounded by their total values x_T^{tot} and x_A^{tot}. The boundedness of r_I can be proved resorting to the comparison principle, as previously done for the system in the absence of titration. □

Equilibria: First we find an expression for $\bar{x}_A$ as a function of $\bar{x}_T$. From $\dot{x}_T + \dot{x}_A = 0$, we obtain:

$$\kappa(x_A^{tot} - \bar{x}_A - \bar{x}_T) = (\delta\bar{x}_T + \nu\bar{x}_A)\bar{r}_I,$$

and from $\dot{x}_T = 0$,

$$\alpha(x_T^{tot} - \bar{x}_T)\bar{x}_A = \delta\bar{x}_T\bar{r}_I.$$

Then

$$\bar{r}_I = \frac{\alpha(x_T^{tot} - \bar{x}_T)\bar{x}_A}{\delta\bar{x}_T} = \frac{\kappa(x_A^{tot} - \bar{x}_A - \bar{x}_T)}{\delta\bar{x}_T + \nu\bar{x}_A}.$$

We obtain a second order polynomial of the following form: $a_x\bar{x}_A^2 + b_x\bar{x}_A + c_x = 0$, where

$$a_x = \frac{\alpha\nu(x_T^{tot} - \bar{x}_T)}{\delta\bar{x}_T}, \quad b_x = \alpha(x_T^{tot} - \bar{x}_T) + \kappa, \quad c_x = -\kappa(x_A^{tot} - \bar{x}_T).$$

Since c_x is always negative, there is a unique positive and acceptable solution:

$$\bar{x}_A(\bar{x}_T) = \frac{-b_x + \sqrt{b_x^2 - 4a_xc_x}}{2a_x}.$$

With this expression we can find $\bar{r}_I$ as a function of $\bar{x}_T$. From $\dot{x}_T = 0$, we obtain:

$$\bar{r}_I(\bar{x}_T) = \frac{\alpha(x_T^{tot} - \bar{x}_T)\bar{x}_A(\bar{x}_T)}{\delta\bar{x}_T}.$$

From $\dot{r}_I = 0$,

$$\bar{u}_I = \frac{1}{\beta}(\delta\bar{x}_T + \phi + \nu\bar{x}_A(\bar{x}_T))\bar{r}_I(\bar{x}_T).$$

Jacobian analysis: The Jacobian matrix becomes:

$$J_I = \begin{bmatrix} -(\alpha\bar{x}_A + \delta\bar{r}_I) & \alpha(x_T^{tot} - \bar{x}_T) & -\delta\bar{x}_T \\ -\kappa + \alpha\bar{x}_A & -[\kappa + \alpha(x_T^{tot} - \bar{x}_T) + \nu\bar{r}_I] & -\nu\bar{x}_A \\ -\delta\bar{r}_I & -\nu\bar{r}_I & -(\delta\bar{x}_T + \nu\bar{x}_A + \phi) \end{bmatrix}.$$

Proposition 6. *The unique equilibrium of* (5)–(7) *is locally stable.*

Proof. Let $p(s) = \det(sI - J_I) = p_3 s^3 + p_2 s^2 + p_1 s + p_0$ be the characteristic polynomial of the linearized system. The polynomial is:

$$p(s) = \det \begin{bmatrix} s + (a+b) & -c & d \\ \kappa - a & s + (\kappa + c + n) & m \\ b & n & s + (d + m + \phi) \end{bmatrix}.$$

We have $p_3 = 1$, $p_2 = a + b + c + d + n + m + \phi + \kappa$,

$$p_1 = \det \begin{bmatrix} -(a+b) & c \\ a - \kappa & -(\kappa + c + n) \end{bmatrix} + \det \begin{bmatrix} -(a+b) & -d \\ -b & -(d + m + \phi) \end{bmatrix} + \det \begin{bmatrix} -(\kappa + c + n) & -m \\ -n & -(d + m + \phi) \end{bmatrix},$$

and finally $p_0 = \det(-J_I)$. Some simple and tedious computations show that $p_k > 0$, $k = 0, 1, 2, 3$. This is necessary, yet not sufficient for stability. According to the Routh–Hurwitz criterion, a polynomial has roots with negative real part if and only if the elements of the first column of the Routh–Hurwitz table are positive. Such a table is:

$$\begin{array}{cc} p_3 & p_1 \\ p_2 & p_0 \\ \frac{p_2 p_1 - p_0 p_3}{p_2} & 0 \\ p_0 & 0 \end{array}$$

Then $p_3 = 1 > 0$, $p_2 > 0$ and $p_0 > 0$. It can be verified analytically that also $p_2 p_1 - p_0 p_3 > 0$, for all positive values of the coefficients. □

1.1.3 Parameter sensitivity

We numerically solved the ODEs describing the behavior of the inhibited module when each reaction rate is varied in a range. ODEs were integrated using MATLAB `ode23s` routine. The results are shown in Fig. S1.

1.2 Analysis of the activated module

1.2.1 Analysis in the absence of direct titration reaction

We recall the model for the activated module:

$$\begin{aligned} \dot{x}_T &= \alpha(x_T^{tot} - x_T) r_A - \delta x_T x_I && (8) \\ \dot{x}_I &= \kappa(x_I^{tot} - x_I - (x_T^{tot} - x_T)) - \delta x_T x_I && (9) \\ \dot{r}_A &= \beta u_A - \alpha(x_T^{tot} - x_T) r_A - \phi r_A && (10) \end{aligned}$$

Assumption 3. *We assume that* $x_I^{tot} \geq x_T^{tot}$.

Additionally, we make an assumption that relates parameters κ, δ, and the total concentrations of target and constitutive inhibitor.

Assumption 4. *We assume that* $x_I^{tot} - x_T^{tot} - \frac{\kappa}{\delta} \geq 0$.

Proposition 7. *For a given concentration of activator source* $\bar{u}_A$*, system* (8)–(10) *has unique equilibrium values* $\bar{x}_T$*,* $\bar{x}_I$ *and* $\bar{r}_A$*. The equilibrium* $\bar{x}_T$ *is a monotonic, strictly increasing function of* $\bar{u}_A$*.*

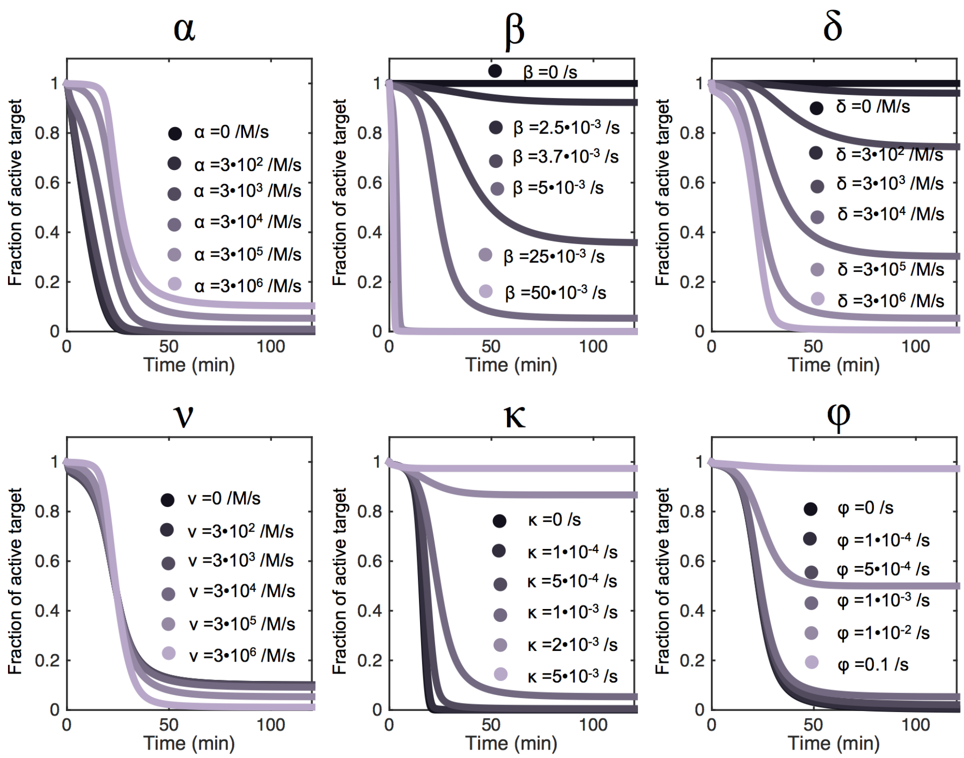

Figure S1: Numerical simulation showing the dependence of the normalized target concentration of the inhibited module $(x_T(t)/x_T^{tot})$ when the reaction rates are varied.

Proof. We begin by finding an expression for $\bar{x}_I$ as a function of $\bar{x}_T$. From $\dot{x}_I = 0$, we obtain:

$$\bar{x}_I(\bar{x}_T) = \frac{\kappa(x_I^{tot} - (x_T^{tot} - \bar{x}_T))}{\delta \bar{x}_T + \kappa}.$$

The equilibrium $\bar{x}_I$ is a monotonic decreasing function of $\bar{x}_T$, as can be seen by checking the sign of the partial derivative:

$$\frac{\partial \bar{x}_I}{\partial \bar{x}_T} = -\frac{\delta}{\kappa} \frac{x_I^{tot} - x_T^{tot} - \frac{\kappa}{\delta}}{(\frac{\delta}{\kappa}\bar{x}_T + 1)^2} \leq 0,$$

in view of Assumption 4.

We continue by finding $\bar{r}_A$ as a function of $\bar{x}_T$. From $\dot{x}_T = 0$, we obtain:

$$\bar{r}_A(\bar{x}_T) = \frac{\delta \bar{x}_T}{\alpha(x_T^{tot} - \bar{x}_T)} \bar{x}_I(\bar{x}_T).$$

With the same approach followed in the proof of Proposition 1, we can show that the equilibrium $\bar{r}_A$ is a monotonic, strictly increasing function of $\bar{x}_T$. Finally, from $\dot{r}_A = 0$ we find:

$$\bar{u}_A = \frac{1}{\beta}\left(\alpha(x_T^{tot} - \bar{x}_T) + \phi\right) \bar{r}_A(\bar{x}_T) = \frac{1}{\beta}(\delta \bar{x}_T \bar{x}_I(\bar{x}_T) + \phi \bar{r}_A(\bar{x}_T)) \doteq w(\bar{x}_T).$$

To identify structural trends between $\bar{u}_A$ and $\bar{x}_T$, we check the sign of the partial derivative $\partial(\bar{x}_T \bar{x}_I(\bar{x}_T))/\partial \bar{x}_T$; some tedious computations show that this partial derivative is always strictly positive, except for $\bar{x}_T = 0$. Therefore $w(\bar{x}_T)$ is a monotonic, strictly increasing function of $\bar{x}_T$; its inverse $\bar{x}_T \doteq k(\bar{u}_I) = w^{-1}(\bar{u}_I)$ is thus also a strictly increasing function. We conclude that the equilibrium $\bar{x}_T$ for a given $\bar{u}_A$ is unique, and so are the other equilibria $\bar{x}_I$ and $\bar{r}_A$. In particular, the higher the concentration of input U_A, the smaller the equilibrium concentration of X_T. □

Proposition 8. *The set*

$$0 \leq x_T \leq x_T^{tot}, \qquad \frac{\kappa}{\delta} \leq x_I \leq x_I^{tot}, \qquad r_A \geq 0 \tag{11}$$

is positively invariant [3] for (8)–(10).

Proof. Since all the variables are non–negative and the variables x_T and x_I cannot exceed their total values, all the constraints are obvious with the exception of $\kappa/\delta \leq x_I$. We show that this constraint cannot be violated: if we start with $x_I(0) \geq \kappa/\delta$, then the constraint is satisfied for all $t > 0$. In fact, due to Assumption 4, for $x_I = \kappa/\delta$ we have

$$\dot{x}_I = \kappa(x_I^{tot} - \frac{\kappa}{\delta} - x_T^{tot} + x_T) - \delta x_T \frac{\kappa}{\delta} = \kappa(x_I^{tot} - x_T^{tot} - \frac{\kappa}{\delta}) \geq 0.$$

□

Proposition 9. *The solutions of system* (8)–(10) *are bounded.*

Proof. Species x_T and x_I are bounded by assumption. The dynamics of the regulator satisfies the inequality $\dot{r}_A(t) \leq \beta u_A^{max} - \phi r_A$. In view of the comparison principle, $r_A(t) \leq r_A(0)e^{-\phi t} + u_A^{max}\beta(1 - e^{-\phi t})/\phi$, which ensures $r_A(t) \leq \max\{r_A(0), u_A^{max}\beta/\phi\}$ at any point in time. □

Proposition 10. *The unique equilibrium of system* (8)–(10) *is locally stable.*

Proof. We follow the proof of Proposition 4. The Jacobian matrix is

$$J_A = \begin{bmatrix} -(\alpha\bar{r}_A + \delta\bar{x}_I) & -\delta x_T & \alpha(x_T^{tot} - \bar{x}_T) \\ \kappa - \delta\bar{x}_I & -(\kappa + \delta\bar{x}_T) & 0 \\ \alpha\bar{r}_A & 0 & -\alpha(x_T^{tot} - \bar{x}_T) - \phi \end{bmatrix}$$

and, since $\delta\bar{x}_I - \kappa > 0$ (Proposition 8), it can be recast as a Metzler matrix by changing sign to its second row and column. As can be shown by direct computation, all the coefficients of the characteristic polynomial $\det(\lambda I - J_A)$ are positive. □

1.2.2 Analysis in the presence of direct titration reactions

When titration reactions are present, the model becomes:

$$\dot{x}_T = \alpha(x_T^{tot} - x_T)r_A - \delta x_T x_I \tag{12}$$
$$\dot{x}_I = \kappa(x_I^{tot} - x_I - (x_T^{tot} - x_T)) - \delta x_T x_I - \nu x_I r_A \tag{13}$$
$$\dot{r}_A = \beta u_A - \alpha(x_T^{tot} - x_T)r_A - \phi r_A - \nu x_I r_A \tag{14}$$

Proposition 11. *The solutions of system* (12)–(14) *are globally bounded.*

Proof. Analogous to that of Proposition 5. □

Equilibria: First we find an expression for $\bar{x}_I$ as a function of $\bar{x}_T$. From $\dot{x}_T - \dot{x}_I = 0$, we obtain:

$$\kappa(x_I^{tot} - \bar{x}_I - (x_T^{tot} - \bar{x}_T)) = (\alpha(x_T^{tot} - \bar{x}_T) + \nu\bar{x}_I)\bar{r}_A,$$

and from $\dot{x}_T = 0$,

$$\alpha(x_T^{tot} - \bar{x}_T)\bar{r}_A = \delta\bar{x}_T\bar{x}_I.$$

Then

$$\bar{r}_A = \frac{\kappa(x_I^{tot} - \bar{x}_I - (x_T^{tot} - \bar{x}_T))}{\alpha(x_T^{tot} - \bar{x}_T) + \nu\bar{x}_I} = \frac{\delta\bar{x}_T\bar{x}_I}{\alpha(x_T^{tot} - \bar{x}_T)}$$

and we obtain a second order polynomial of the following form: $a_x\bar{x}_I^2 + b_x\bar{x}_I + c_x = 0$, where

$$a_x = \frac{\delta\nu\bar{x}_T}{\alpha(x_T^{tot} - \bar{x}_T)}, \quad b_x = \alpha\bar{x}_T + \kappa, \quad c_x = -\kappa(x_I^{tot} - (x_T^{tot} - \bar{x}_T)).$$

Since c_x is always negative, the unique positive and acceptable solution is

$$\bar{x}_I(\bar{x}_T) = \frac{-b_x + \sqrt{b_x^2 - 4a_xc_x}}{2a_x}.$$

Then, we find $\bar{r}_A$ as a function of $\bar{x}_T$. From $\dot{x}_T = 0$, we obtain:

$$\bar{r}_A(\bar{x}_T) = \frac{\delta\bar{x}_T\bar{x}_I(\bar{x}_T)}{\alpha(x_T^{tot} - \bar{x}_T)}.$$

Finally from $\dot{r}_A = 0$:

$$\bar{u}_A = \frac{1}{\beta}(\alpha(x_T^{tot} - \bar{x}_T) + \phi + \nu\bar{x}_I(\bar{x}_T))\bar{r}_A(\bar{x}_T).$$

Jacobian analysis: The Jacobian matrix is now:

$$J_A = \begin{bmatrix} -(\alpha\bar{r}_A + \delta\bar{x}_I) & -\delta\bar{x}_T & \alpha(x_T^{tot} - \bar{x}_T) \\ \kappa - \delta\bar{x}_I & -(\kappa + \delta\bar{x}_T + \nu\bar{r}_A) & -\nu\bar{x}_I \\ \alpha\bar{r}_A & -\nu\bar{r}_A & -(\alpha(x_T^{tot} - \bar{x}_T) + \nu\bar{x}_I + \phi) \end{bmatrix}$$

Proposition 12. *The unique equilibrium of* (12)–(14) *is locally stable.*

Proof. Analogous to that of Proposition 6. □

1.2.3 Parameter sensitivity

Fig. S2 shows the behavior of the activated module when each reaction rates is varied in a range. ODEs were integrated using MATLAB `ode23s` routine.

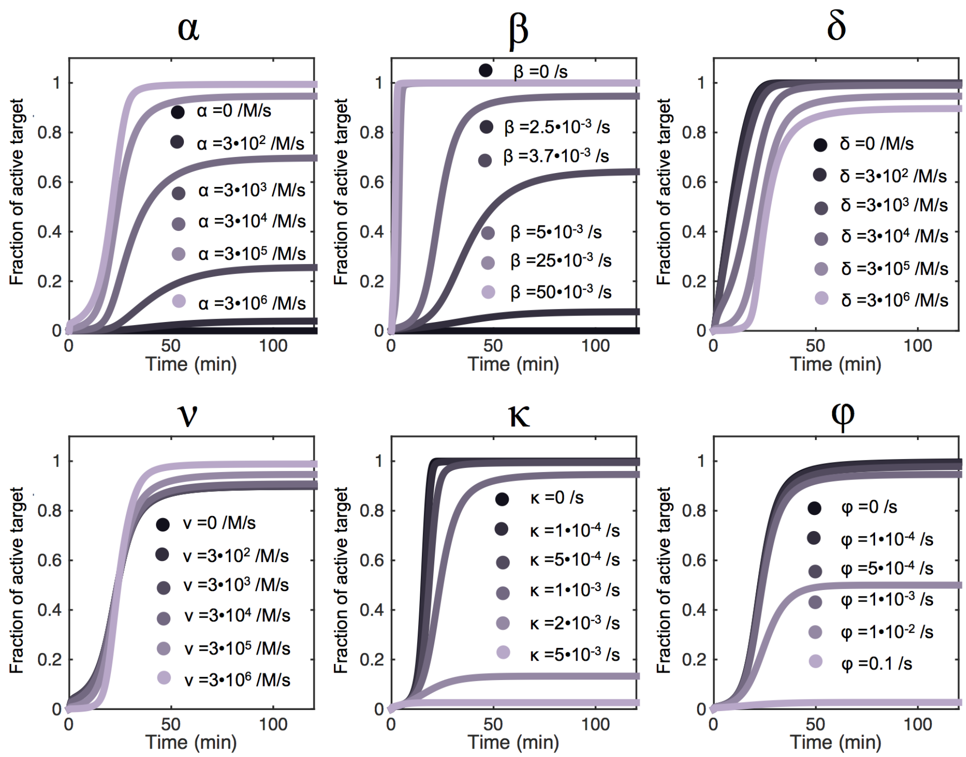

Figure S2: Numerical simulation showing the dependence of the normalized target concentration of the activated module ($x_T(t)/x_T^{tot}$) when the reaction rates are varied.

2 Oscillator

As discussed in the main text, we build an oscillator via the feedback interconnection of an inhibited module and an activated module. The reactions occurring in the system are:

	Activated subsystem	**Inhibited subsystem**
Activation:	$X_{R,A} + Z_T^* \xrightarrow{\alpha_z} Z_T + Z_I^*$	$X_A + X_T^* \xrightarrow{\alpha_x} X_T$
Output production:	$Z_T \xrightarrow{\beta_z} Z_{R,I} + Z_T$	$X_T \xrightarrow{\beta_x} X_{R,A} + X_T$
Inhibition:	$Z_T + Z_I \xrightarrow{\delta_z} Z_T^*$	$Z_{R,I} + X_T \xrightarrow{\delta_x} X_T^* + X_A^*$
Conversion:	$Z_I^* \xrightarrow{\kappa_z} Z_I$	$X_A^* \xrightarrow{\kappa_x} X_A$
Direct titration:	$X_{R,A} + Z_I \xrightarrow{\nu_z} Z_I^*$	$Z_{R,I} + X_A \xrightarrow{\nu_x} X_A^*.$
Degradation:	$Z_{R,I} \xrightarrow{\phi_z} 0$	$X_{R,A} \xrightarrow{\phi_x} 0$

The regulators interconnecting the modules are $x_{R,A}$, which is the output of the inhibited module and works as an activator for the activated module, and $z_{R,I}$, which is the output of the activated module and works as an inhibitor for the inhibited module. We recall that we assume mass conservation for species Z_T, Z_I, X_T, and X_A: $z_T^{tot} = z_T + z_T^*$, $z_I^{tot} = z_I + z_I^* + z_T^*$, $x_T^{tot} = x_T + x_T^*$, $x_A^{tot} = x_A + x_A^* + x_T$. Using the law of mass action we derive the differential equations:

$$\dot{z}_T = \alpha_z(z_T^{tot} - z_T)x_{R,A} - \delta_z z_T\, z_I, \tag{15}$$
$$\dot{z}_I = \kappa_z(z_I^{tot} - z_I - (z_T^{tot} - z_T)) - \delta_z z_T\, z_I - \nu_z x_{R,A}\, z_I, \tag{16}$$
$$\dot{x}_{R,A} = \beta_x x_T - \alpha_z(z_T^{tot} - z_T)\, x_{R,A} - \nu_z x_{R,A}\, z_I - \phi_x x_{R,A}, \tag{17}$$
$$\dot{x}_T = \alpha_x(x_T^{tot} - x_T)x_A - \delta_x x_T\, z_{R,I}, \tag{18}$$
$$\dot{x}_A = \kappa_x(x_A^{tot} - x_A - x_T) - \alpha_x(x_T^{tot} - x_T)\, x_A - \nu_x x_A\, z_{R,I}, \tag{19}$$
$$\dot{z}_{R,I} = \beta_z z_T - \delta_x x_T\, z_{R,I} - \nu_x x_A\, z_{R,I} - \phi_z z_{R,I}. \tag{20}$$

Throughout our analysis, we assume that $z_I^{tot} \geq z_T^{tot}$ and $x_A^{tot} \geq x_T^{tot}$.

As a preliminary result, we notice that the interconnection does not change the boundedness property of the solution.

Proposition 13. *The solutions of system* (15)–(20) *are bounded.*

Proof. The proposition follows from the fact that each subsystem has bounded solution for bounded inputs. Then we notice that the inhibited subsystem (x_T–x_A–$z_{R,I}$) has input $\beta_z z_T \leq \beta_z z_T^{tot}$, which is bounded, while the activated subsystem (z_T–z_I–$x_{R,A}$) has input $\beta_x x_T \leq \beta_x x_T^{tot}$, bounded as well. □

2.1 Analysis in the absence of direct titration reactions

2.1.1 Equilibrium conditions

In this section, we consider the oscillatory system (15)–(20) in the absence of titration reactions, *i.e.*, with $\nu_x = \nu_z = 0$. We derive equilibrium conditions that are consistent with those derived for the inhibited and activated module. First, we find an expression for $\bar{x}_T$ as a function of $\bar{z}_T$. From $\dot{z}_I = 0$, we obtain:

$$\bar{z}_I(\bar{z}_T) = \frac{\kappa_z(z_I^{tot} - (z_T^{tot} - \bar{z}_T))}{\delta_z \bar{z}_T + \kappa_z}.$$

From $\dot{z}_T = 0$, we obtain:

$$\bar{x}_{R,A}(\bar{z}_T) = \frac{\delta_z \bar{z}_T\, \bar{z}_I(\bar{z}_T)}{\alpha_z(z_T^{tot} - \bar{z}_T)}.$$

From $\dot{x}_{R,A} = 0$,

$$\bar{x}_T = \frac{1}{\beta_x}(\alpha_z(z_T^{tot} - \bar{z}_T) + \phi_x)\bar{x}_{R,A}(\bar{z}_T) \doteq w(\bar{z}_T).$$

As shown in Proposition 1, $\bar{x}_T$ is a monotonically decreasing function of $\bar{z}_T$.

We now find an expression for $\bar{z}_T$ as a function of $\bar{x}_T$. From $\dot{x}_A = 0$, we obtain:

$$\bar{x}_A(\bar{x}_T) = \frac{\kappa_x(x_A^{tot} - \bar{x}_T)}{\alpha_x(x_T^{tot} - \bar{x}_T) + \kappa_x}.$$

From $\dot{x}_T = 0$, we obtain:

$$\bar{z}_{R,I}(\bar{x}_T) = \frac{\alpha_x(x_T^{tot} - \bar{x}_T)\bar{x}_A(\bar{x}_T)}{\delta_x\bar{x}_T}.$$

From $\dot{z}_{R,I} = 0$,

$$\bar{z}_T = \frac{1}{\beta_z}(\delta_x\bar{x}_T + \phi_z)\bar{z}_{R,I}(\bar{x}_T) \doteq h(\bar{x}_T).$$

As shown in Proposition 7, $\bar{z}_T$ is a monotonically increasing function of $\bar{x}_T$. The functions $h(\bar{x}_T)$ and $w(\bar{z}_T)$ found above, because of their opposite trend, can admit a single intersection in the plane $(\bar{x}_T, \bar{z}_T)$. These equilibrium conditions will be used to find numerically or graphically the unique equilibrium point of the system.

2.1.2 Structural oscillations

The equilibrium conditions derived earlier show that there exists a single equilibrium, around which we linearize the system. It is convenient to change the sign of some of the variables: $-z_I$, $-x_T$, $-x_A$. The Jacobian matrix of system (15)–(20) with $\nu_z = \nu_x = 0$ becomes:

$$J = \left[\begin{array}{ccc|ccc} -\alpha_z\bar{x}_{R,A} - \delta_z\bar{z}_I & \delta_z\bar{z}_T & \alpha_z(z_T^{tot} - \bar{z}_T) & 0 & 0 & 0 \\ -\kappa_z + \delta_z\bar{z}_I & -\kappa_z - \delta_z\bar{z}_T & 0 & 0 & 0 & 0 \\ \alpha_z\bar{x}_{R,A} & 0 & -\alpha_z(z_T^{tot} - \bar{z}_T) - \phi_x & 0 & -\beta_x & 0 \\ \hline \beta_z & 0 & 0 & -\delta_x\bar{x}_T - \phi_z & \delta_x\bar{z}_{R,I} & 0 \\ 0 & 0 & 0 & \delta_x\bar{x}_T & -\alpha_x\bar{x}_A - \delta_x\bar{z}_{R,I} & \alpha_x(x_T^{tot} - \bar{x}_T) \\ 0 & 0 & 0 & 0 & -\kappa_x + \alpha_x\bar{x}_A & -\kappa_x - \alpha_x(x_T^{tot} - \bar{x}_T) \end{array}\right] \tag{21}$$

We call *strong candidate oscillator* [1, 2] a system which can be locally unstable exclusively due to the existence of complex conjugate eigenvalues with nonnegative real part (in other words, the system does not admit real nonnegative eigenvalues).

Proposition 14. *Under Assumptions 1, 2, 3, and 4, system* (15)–(20) *is a strong candidate oscillator.*

Proof. The computation of the characteristic polynomial $\det(\lambda I - J)$ reveals that all the coefficients are positive (note that we assume $-\kappa_z + \delta_z\bar{z}_I > 0$ and $-\kappa_x + \alpha_x\bar{x}_A > 0$). A polynomial with positive coefficients cannot have nonnegative real roots. □

2.1.3 Linear analysis

The Jacobian (21) clearly shows that the system is the feedback interconnection of two subsystems of the third order. To simplify the notation we define:

$$\begin{array}{rclcrcl} a_1 & \doteq & \delta_z\bar{z}_T, & & a_2 & \doteq & \delta_x\bar{x}_T, \\ b_1 & \doteq & \delta_z\bar{z}_I, & & b_2 & \doteq & \delta_x\bar{z}_{R,I}, \\ c_1 & \doteq & \alpha_z\bar{x}_{R,A}, & & c_2 & \doteq & \alpha_x\bar{x}_A, \\ d_1 & \doteq & \alpha_z(z_T^{tot} - \bar{z}_T), & & d_2 & \doteq & \alpha_x(x_T^{tot} - \bar{x}_T), \\ e_1 & \doteq & \kappa_z, & & e_2 & \doteq & \kappa_x, \\ h_1 & \doteq & \phi_x, & & h_2 & \doteq & \phi_z, \end{array}$$

Then, defining vectors $\xi_1 = \begin{bmatrix} z_T & -z_I & x_{R,A} \end{bmatrix}^\top$ and $\xi_2 = \begin{bmatrix} z_{R,I} & -x_T & -x_A \end{bmatrix}^\top$, the linearized system can be rewritten as the feedback interconnection of:

$$\dot{\xi}_1 = A_1\xi_1 + \beta_x B_1 \omega_2, \quad \omega_1 = C_1\xi_1,$$

and

$$\dot{\xi}_2 = A_2\xi_2 + \beta_z B_2 \omega_1, \quad \omega_2 = C_2\xi_2,$$

where:

$$A_1 = \begin{bmatrix} -(c_1+b_1) & a_1 & d_1 \\ -e_1+b_1 & -(e_1+a_1) & 0 \\ c_1 & 0 & -(d_1+h_1) \end{bmatrix}, \quad B_1 = \begin{bmatrix} 0 \\ 0 \\ -1 \end{bmatrix}, \quad C_1 = \begin{bmatrix} 1 & 0 & 0 \end{bmatrix} \tag{22}$$

and

$$A_2 = \begin{bmatrix} -(a_2+h_2) & b_2 & 0 \\ a_2 & -(c_2+b_2) & d_2 \\ 0 & -e_2+c_2 & -(e_2+d_2) \end{bmatrix}, \quad B_2 = \begin{bmatrix} 1 \\ 0 \\ 0 \end{bmatrix}, \quad C_2 = \begin{bmatrix} 0 & 1 & 0 \end{bmatrix}. \tag{23}$$

By applying the Laplace transform method, we can obtain an input-output representation of the two subsystems in terms of their transfer functions[1]: $F_1(s) = -\frac{n_1(s)}{d_1(s)}$ and $F_2(s) = \frac{n_2(s)}{d_2(s)}$. Since the overall feedback loop is negative and all the coefficients of the numerator and denominator polynomials $n_1(s)$, $d_1(s)$, $n_2(s)$, $d_2(s)$ are positive, the closed–loop characteristic polynomial also has positive coefficients. Therefore it cannot admit non-negative real roots, as stated in the following proposition, hence the system is a strong candidate oscillator.

Proposition 15. *Consider system* (15)–(20)*, where $\nu_x = \nu_z = 0$, linearized around its only equlibrium point. Its characteristic polynomial has no real nonnegative roots. If instability occurs, it is oscillatory, namely due to complex roots with positive real part.*

The fact that the system is a candidate oscillator in the strong sense does not mean that the system oscillates for any choice of the parameters. In fact, numerical simulations show that oscillations occur only in a limited region in the plane defined by β_z and β_x.

It is worth noticing that in the absence of titration reactions, being $c_2 - e_2$ and $b_1 - e_1$ positive quantities, the system is the negative feedback interconnection of two monotone subsystems, associated with the modules (see [1, 2] and the references therein): this structurally explains its oscillatory nature.

2.2 Analysis in the presence of direct titration reactions

2.2.1 Equilibrium conditions

We consider in this section the oscillatory system (15)–(20) in the presence of non–zero ν_x and ν_z. We begin by finding two expressions of $\bar{x}_{R,A}$ as a function of the other variables. This can be done by setting $\dot{z}_T - \dot{z}_I = 0$ and $\dot{z}_T = 0$. Then, we equate the two expressions for $\bar{x}_{R,A}$ and we achieve:

$$\frac{\delta_z \bar{z}_T\, \bar{z}_I}{\alpha_z(z_T^{tot} - \bar{z}_T)} = \frac{\kappa_z(z_I^{tot} - \bar{z}_I - (z_T^{tot} - \bar{z}_T))}{\alpha_z(z_T^{tot} - \bar{z}_T) + \nu_z \bar{z}_I},$$

[1] Given a linear system with an input $u(t)$ and an output $y(t)$, its transfer function $F(s)$ is the ratio between the Laplace transform of the output and the Laplace transform of the input: $F(s) = \frac{Y(s)}{U(s)}$.

which defines a relationship between $\bar{z}_T$ and $\bar{z}_I$ at steady state. The equilibrium $\bar{z}_I$ can thus be derived as the solution of the second order equation $a_z\bar{z}_I^2 + b_z\bar{z}_I + c_z = 0$, where $a_z = \left(\frac{\delta_z\nu_z}{\alpha_z}\right)\frac{\bar{z}_T}{z_T^{tot}-\bar{z}_T}$, $\quad b_z = (\delta_z\bar{z}_T + \kappa_z)$ and $c_z = -\kappa_z(z_I^{tot} - (z_T^{tot} - \bar{z}_T))$. Assuming $z_I^{tot} > z_T^{tot}$, since $a_z c_z < 0$, the only admissible positive solution is:

$$\bar{z}_I(\bar{z}_T) = \frac{-b_z + \sqrt{b_z^2 - 4a_z c_z}}{2a_z}.$$

Then,

$$\bar{x}_{R,A} = \frac{\delta_z\bar{z}_T\ \bar{z}_I(\bar{z}_T)}{\alpha_z(z_T^{tot} - \bar{z}_T)}.$$

Finally, we can find $\bar{x}_T$ as a function of $\bar{z}_T$, by setting $\dot{x}_{R,A} = 0$:

$$\bar{x}_T = \frac{1}{\beta_x}(\alpha_z(z_T^{tot} - \bar{z}_T) + \phi_x + \nu_z\bar{z}_I(\bar{z}_T))\bar{x}_{R,A}(\bar{z}_T).$$

We can proceed similarly to derive $\bar{z}_T$ as a function of $\bar{x}_T$. Setting $\dot{x}_T + \dot{x}_A = 0$ and $\dot{x}_T = 0$, we find two different expressions for $\bar{z}_{R,I}$. Equating these expressions we find:

$$\frac{\alpha_x(x_T^{tot} - \bar{x}_T)\,\bar{x}_A}{\delta_x\bar{x}_T} = \frac{\kappa_x(x_A^{tot} - \bar{x}_A - \bar{x}_T)}{\delta_x\bar{x}_T + \nu_x\bar{x}_A},$$

so we can isolate the relationship between $\bar{x}_T$ and $\bar{x}_A$ at steady state. As done before, we derive the equilibrium $\bar{x}_A$ as the solution of the second order equation $a_x\bar{x}_A^2 + b_x\bar{x}_A + c_x = 0$, where $a_x = \left(\frac{\alpha_x\nu_x}{\delta_x}\right)\frac{x_T^{tot}-\bar{x}_T}{\bar{x}_T}$, $b_x = \left(\alpha_x(x_T^{tot} - \bar{x}_T) + \kappa_x\right)$ and $c_x = -\kappa_x(x_A^{tot} - \bar{x}_T)$. Assuming $x_A^{tot} > x_T^{tot}$, since again $a_x c_x < 0$, the only admissible positive solution is:

$$\bar{x}_A(\bar{x}_T) = \frac{-b_x + \sqrt{b_x^2 - 4a_x c_x}}{2a_x}.$$

Then,

$$\bar{z}_{R,I} = \frac{\alpha_x(x_T^{tot} - \bar{x}_T)\,\bar{x}_A(\bar{x}_T)}{\delta_x\bar{x}_T}.$$

Finally, we can find $\bar{z}_T$ as a function of $\bar{x}_T$, by setting $\dot{z}_{R,I} = 0$:

$$\bar{z}_T = \frac{1}{\beta_z}(\delta_x\bar{x}_T + \phi_z + \nu_x\bar{x}_A(\bar{x}_T))\bar{z}_{R,I}(\bar{x}_T).$$

Once we find the only admissible equilibrium values $\bar{z}_T$, $\bar{z}_I$, $\bar{x}_T$ and $\bar{x}_A$ we can find $\bar{z}_{R,I}$ and $\bar{x}_{R,A}$.

$$\dot{z}_{R,I} = 0 \quad\Longrightarrow\quad \bar{z}_{R,I} = \frac{\beta_z\bar{z}_T}{\delta_x\bar{x}_T + \nu_x\bar{x}_A + \phi_z},$$

$$\dot{x}_{R,A} = 0 \quad\Longrightarrow\quad \bar{x}_{R,A} = \frac{\beta_x\bar{x}_T}{\alpha_z(z_T^{tot} - \bar{z}_T) + \nu_z\bar{z}_I + \phi_x}.$$

2.2.2 Structural oscillations

As done before, we change the sign to some of the variables, which become $-z_I$, $-x_T$, $-x_A$; the Jacobian of the system in the presence of titration reactions becomes matrix J_ν

$$J_\nu = \tag{24}$$

$$\left[\begin{array}{ccc|ccc}
-\alpha_z\bar{x}_{R,A} - \delta_z\bar{z}_I & \delta_z\bar{z}_T & \alpha_z(z_T^{tot} - \bar{z}_T) & 0 & 0 & 0 \\
-\kappa_z + \delta_z\bar{z}_I & -\kappa_z - \delta_z\bar{z}_T - \nu_z\bar{x}_{R,A} & \nu_z\bar{z}_I & 0 & 0 & 0 \\
\alpha_z\bar{x}_{R,A} & \nu_z\bar{x}_{R,A} & -\alpha_z(z_T^{tot} - \bar{z}_T) - \nu_z\bar{z}_I - \phi_x & 0 & -\beta_x & 0 \\
\hline
\beta_z & 0 & 0 & -\delta_x\bar{x}_T - \nu_x\bar{x}_A - \phi_z & \delta_x\bar{z}_{R,I} & \nu_x\bar{z}_{R,I} \\
0 & 0 & 0 & \delta_x\bar{x}_T & -\alpha_x\bar{x}_A - \delta_x\bar{z}_{R,I} & \alpha_x(x_T^{tot} - \bar{x}_T) \\
0 & 0 & 0 & \nu_x\bar{x}_A & -\kappa_x + \alpha_x\bar{x}_A & -\kappa_x - \alpha_x(x_T^{tot} - \bar{x}_T) - \nu_x\bar{z}_{R,I}
\end{array}\right]$$

2.2.3 Linear analysis

As done earlier, we simplify the notation defining:

$$
\begin{array}{rclcrcl}
a_1 & \doteq & \delta_z \bar{z}_T, & \qquad & a_2 & \doteq & \delta_x \bar{x}_T, \\
b_1 & \doteq & \delta_z \bar{z}_I, & & b_2 & \doteq & \delta_x \bar{z}_{R,I}, \\
c_1 & \doteq & \alpha_z \bar{x}_{R,A}, & & c_2 & \doteq & \alpha_x \bar{x}_A, \\
d_1 & \doteq & \alpha_z (z_T^{tot} - \bar{z}_T), & & d_2 & \doteq & \alpha_x (x_T^{tot} - \bar{x}_T), \\
e_1 & \doteq & \kappa_z, & & e_2 & \doteq & \kappa_x, \\
f_1 & \doteq & \nu_z \bar{x}_{R,A}, & & f_2 & \doteq & \nu_x \bar{x}_A, \\
g_1 & \doteq & \nu_z \bar{z}_I, & & g_2 & \doteq & \nu_x \bar{z}_{R,I}. \\
h_1 & \doteq & \phi_x, & & h_2 & \doteq & \phi_z
\end{array}
$$

Then, defining $\xi_1 = \begin{bmatrix} z_T & -z_I & -x_{R,A} \end{bmatrix}^\top$ and $\xi_2 = \begin{bmatrix} z_{R,I} & -x_T & -x_A \end{bmatrix}^\top$, the linearized system can be rewritten as the feedback interconnection of two linear systems:

$$\dot{\xi}_1 = A_1 \xi_1 + \beta_x B_1 \omega_2, \quad \omega_1 = C_1 \xi_1,$$

and

$$\dot{\xi}_2 = A_2 \xi_2 + \beta_z B_2 \omega_1, \quad \omega_2 = C_2 \xi_2,$$

where:

$$A_1 = \begin{bmatrix} -(c_1 + b_1) & a_1 & d_1 \\ -e_1 + b_1 & -(e_1 + a_1 + f_1) & g_1 \\ c_1 & f_1 & -(d_1 + g_1 + h_1) \end{bmatrix}, \quad B_1 = \begin{bmatrix} 0 \\ 0 \\ -1 \end{bmatrix}, \quad C_1 = \begin{bmatrix} 1 & 0 & 0 \end{bmatrix} \tag{25}$$

and

$$A_2 = \begin{bmatrix} -(a_2 + f_2 + h_2) & b_2 & g_2 \\ a_2 & -(c_2 + b_2) & d_2 \\ f_2 & -e_2 + c_2 & -(e_2 + d_2 + g_2) \end{bmatrix}, \quad B_2 = \begin{bmatrix} 1 \\ 0 \\ 0 \end{bmatrix}, \quad C_2 = \begin{bmatrix} 0 & 1 & 0 \end{bmatrix}. \tag{26}$$

The transfer functions associated with the two subsystems (25) and (26) are:

$$F_1(s) = -\frac{d_1 s + a_1 g_1 + d_1 e_1 + d_1 a_1 + d_1 f_1}{p_1(s)}$$

and

$$F_2(s) = \frac{a_2 s + a_2 e_2 + a_2 d_2 + a_2 g_2 + d_2 f_2}{p_2(s)},$$

where $p_1(s)$ and $p_2(s)$ are third order polynomials having positive coefficients.

As in the case without titration reactions, the interconnection of the two subsystems is a negative feedback loop. The polynomials of the numerator and denominator of both the transfer functions have positive coefficients. As a consequence, the closed–loop characteristic polynomial has positive coefficients. A polynomial with positive coefficients cannot have nonnegative real roots. Therefore, instability can occur only with complex conjugate poles with positive real part, thus it can only be oscillatory. This confirms the result of our previous structural analysis.

Proposition 16. *Consider system* (15)–(20)*, where* $\nu_x > 0$*,* $\nu_z > 0$*, linearized around its only equlibrium point. Its characteristic polynomial has no real nonnegative roots. Instability can only occur due to complex roots with positive real part.*

2.3 Numerical simulations

2.3.1 Randomized parameter search

Table S1: Nominal parameters for the oscillator model (15)–(20)

Rate	Value	Rate	Value
α_z (/M/s)	$75 \cdot 10^3$	α_x (/M/s)	$3 \cdot 10^5$
δ_z (/M/s)	$3 \cdot 10^5$	δ_x (/M/s)	$3 \cdot 10^5$
ν_z (/M/s)	$3 \cdot 10^5$	ν_x (/M/s)	$3 \cdot 10^5$
β_z (/s)	$5 \cdot 10^{-3}$	β_x (/s)	$2 \cdot 10^{-2}$
κ_z (/s)	$1 \cdot 10^{-3}$	κ_x (/s)	$1 \cdot 10^{-3}$
ϕ_z (/s)	$1 \cdot 10^{-3}$	ϕ_x (/s)	$1 \cdot 10^{-3}$
z_T^{tot} (nM)	250	x_T^{tot} (nM)	120
z_I^{tot} (nM)	700	x_A^{tot} (nM)	300

We numerically searched parameters that yield an oscillatory behavior in model (15)–(20). We generated random parameter values starting from the nominal parameter set listed on Table S1. We generated several hundreds of random parameter sets; reaction rates were varied in the range from 10^{-3} to 10^3 times their nominal values; z_T^{tot}, z_I^{tot}, x_T^{tot} and x_A^{tot} were changed in the range from 10^{-1} to 10 times their nominal values . For each parameter set, the differential equations (15)–(20) are solved using the deterministic integrator RADAU, included in the software PyDSTool [4]. A parameter set is classified as "oscillatory" if at least 3 oscillations are detected, their average period is beween $0.5h$ and $10h$, and their average amplitude is larger than 10 nM. Each trajectory was integrated to have a duration of 20 h. Our method follows the approach proposed in [5].

Period and amplitude were computed by identifying minima and maxima of oscillations, as shown in the inset of Fig. S3. For each three consecutive points of a trajectory, we define $d1$ and $d2$ as shown in Fig. S3 B: $d1 = p_n - p_{n-1}$ and $d2 = p_n - p_{n+1}$. If the product $d1 \cdot d2$ is positive and $d1$ is positive, then p_n is classified as a local maximum; if $d1$ is negative, then p_n is classified as a local minimum. Period and amplitude are computed from the identified maxima and minima, as sketched in Fig. S3 C. Period and amplitude are averaged over all the different measured peaks and wells and compared to the aforementioned thresholds.

Fig. S3 A shows the correlations among pairs of parameters that yield oscillations in the absence of direct titration reactions. Fig. S4 shows the results in the presence of titration reactions: the probability of oscillation is significantly increased. Some of the correlation plots show clear patterns. For example the plots clearly show that z_I^{tot} should be larger than z_T^{tot} and x_A^{tot} larger than x_T^{tot}. Both β_z and β_x should be sufficiently large (relative to the nominal value).

2.3.2 Classification of dynamic behaviors in a region of the parameter space

We now classify equilibria as oscillatory or not by checking the eigenvalues of the Jacobian matrix (24) for a given parameter set (we recall that the system has a unique equilibrium for arbitrary choices of parameters). Starting from the nominal parameters listed in Table S1; all parameters were changed in the range from 10^{-1} to 10 times their nominal values. Two parameters were varied at a time, while others were held constant, to generate each subplot of Fig. S5 and S6. Equilibria were computed as the intersections of the analytical equilibrium expressions found at Section 2.2.1. Then, the stability properties of the equilibrium points are computed by finding the eigenvalues of the Jacobian evaluated at the equilibrium. When the Jacobian has at least one pair of complex conjugate eigenvalues with positive real part, it is classified as oscillatory; otherwise, it is classified as non-oscillatory and thus stable.

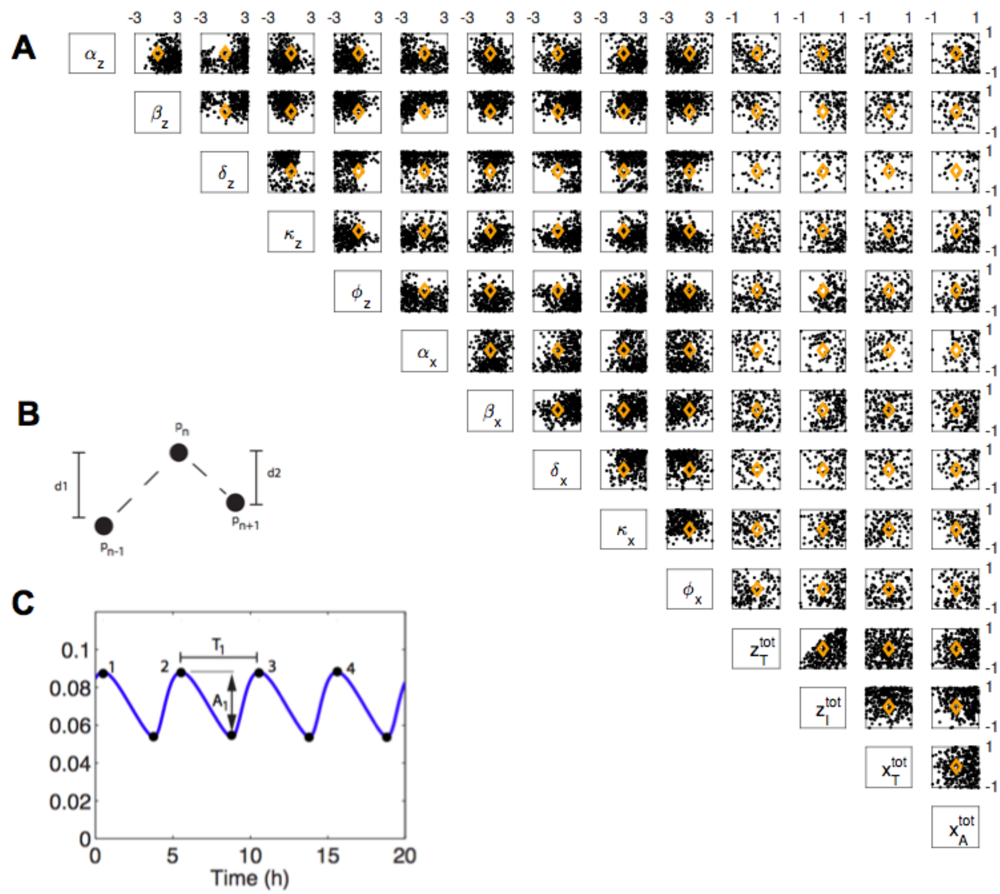

Figure S3: A: Absence of direct titration reactions. Correlation between randomly chosen parameters that yield oscillatory behavior. B: Points required for the identification of period and amplitude. C: Period and amplitude were measured as shown here and averaged over the trajectory.

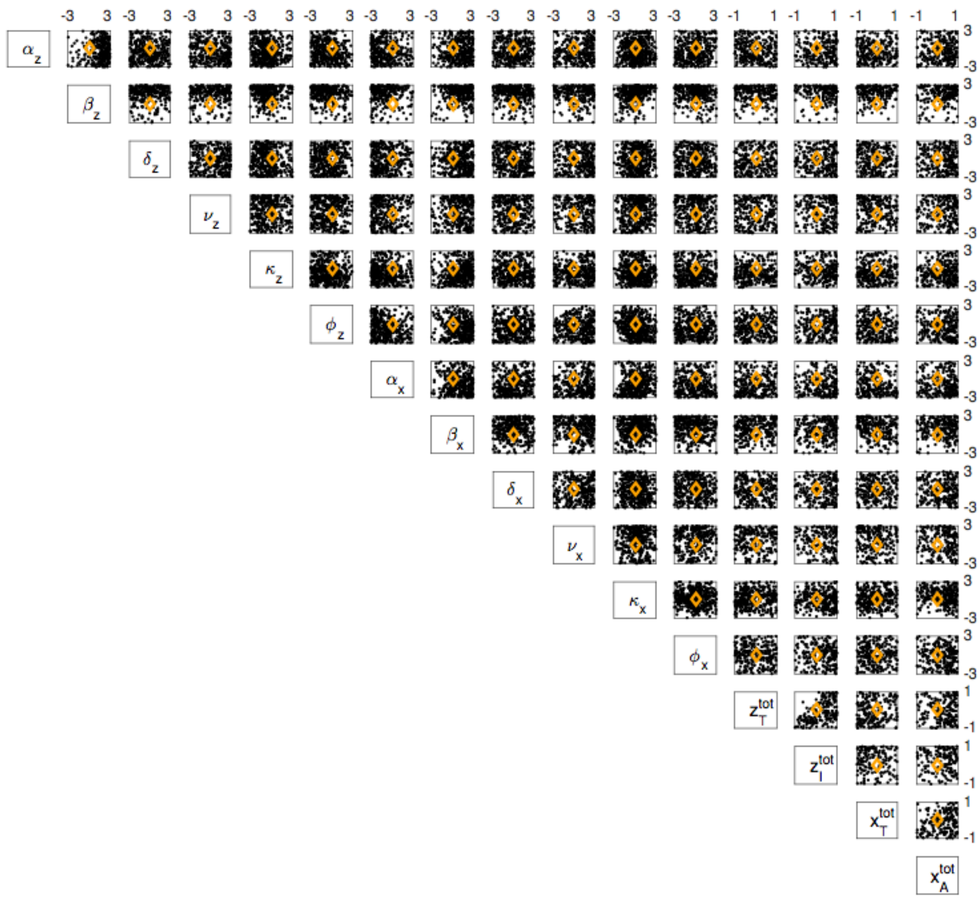

Figure S4: Presence of direct titration reactions. Correlation between randomly chosen parameters that yield oscillatory behavior. The probability of oscillation for a randomly chosen set of parameters significantly increases relative to Fig. S3.

We summarize our results in Fig. S5 and S6, which show the influence of the parameters on the stability properties of the unique equilibrium of the system; we consider the case where titration reactions are absent (Fig. S5), and the case where titration reactions are comparable to the inhibition/activation rates of the regulators that interconnect the two modules (Fig. S6). The classification is color coded as follows: points where at least one pair of eigenvalues is complex with positive real part are shown in orange color; points at which we find real and negative eigenvalues or complex with negative real part are shown in blue color. These plots show some linear correlations among parameters that yield oscillations: (β_z, x_A^{tot}) and (β_x, z_I^{tot}) are positively correlated; (κ_x, x_A^{tot}) and (κ_z, x_I^{tot}) are negatively correlated in order to guarantee an oscillatory behavior. The presence of titration reactions considerably expands the oscillatory regions for all pairs of parameters.

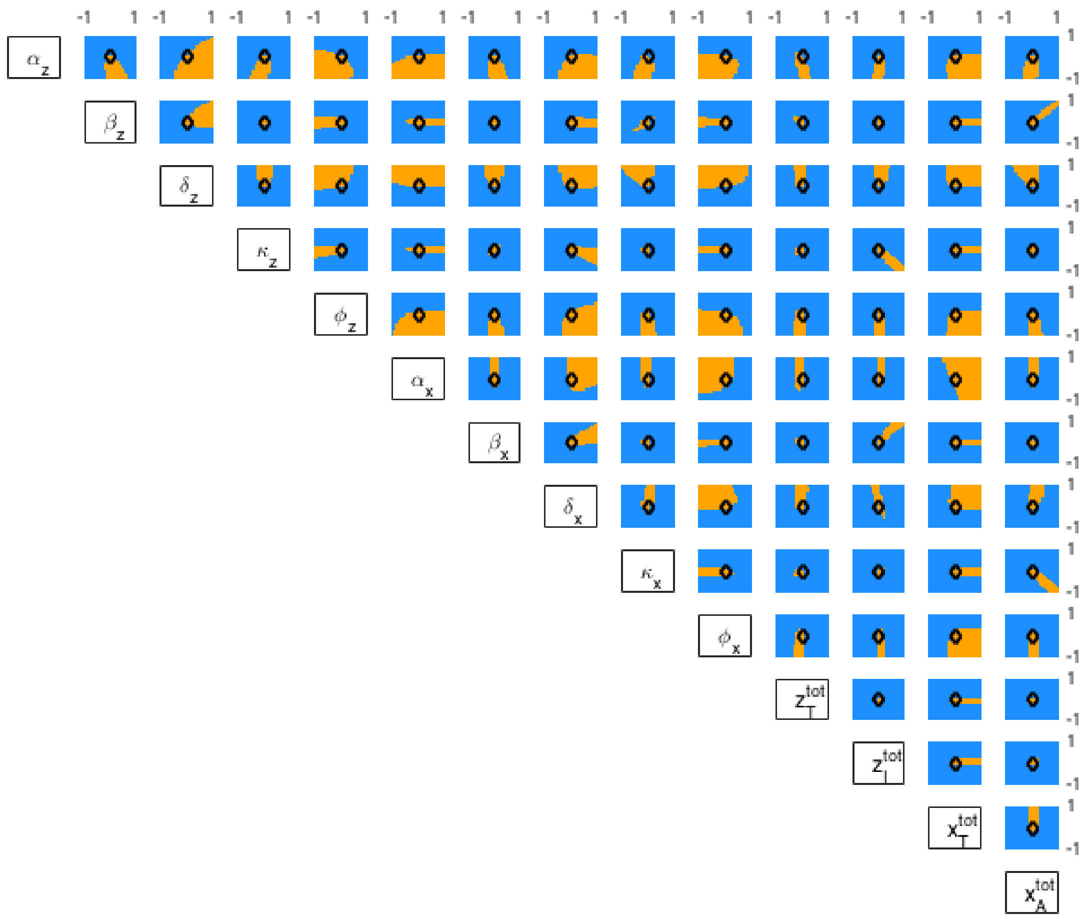

Figure S5: Absence of direct titration reactions: the log plots show the influence of variations of parameters on the stability of the equilibrium. Each parameter was varied between one tenth to ten times the nominal value (black diamond). Orange regions are oscillatory; blue regions indicate stable equilibria.

Figure S6: Presence of direct titration reactions: the log plots show the influence of variations of parameters on the stability of the equilibrium. Each parameter was varied between one tenth to ten times the nominal value (black diamond). Orange regions are oscillatory; blue regions indicate stable equilibria. The orange (oscillatory) regions are considerably larger than those in Fig. S5, where titration is absent.

3 Bistable system

We build a bistable system via the feedback interconnection of two inhibited modules. The reactions describing the bistable system are:

	Subsystem 1	**Subsystem 2**
Activation:	$Z_A + Z_T^* \xrightarrow{\alpha_z} Z_T$	$X_A + X_T^* \xrightarrow{\alpha_x} X_T$
Output production:	$Z_T \xrightarrow{\beta_z} Z_{R,I} + Z_T$	$X_T \xrightarrow{\beta_x} X_{R,I} + X_T$
Inhibition:	$X_{R,I} + Z_T \xrightarrow{\delta_z} Z_T^* + Z_A^*$	$Z_{R,I} + X_T \xrightarrow{\delta_x} X_T^* + X_A^*$
Direct titration:	$X_{R,I} + Z_A \xrightarrow{\nu_z} Z_A^*$	$Z_{R,I} + X_A \xrightarrow{\nu_x} X_A^*$
Conversion:	$Z_A^* \xrightarrow{\kappa_z} Z_A$	$X_A^* \xrightarrow{\kappa_x} X_A$
Degradation:	$Z_{R,I} \xrightarrow{\phi_z} 0$	$X_{R,I} \xrightarrow{\phi_x} 0$

The regulators interconnecting the modules are $x_{R,I}$ and $z_{R,I}$; both work as inhibitors. We assume mass conservation for species Z_T, Z_A, X_T, and X_A: $z_T^{tot} = z_T + z_T^*$, $z_A^{tot} = z_A + z_A^* + z_T$, $x_T^{tot} = x_T + x_T^*$, $x_A^{tot} = x_A + x_A^* + x_T$. The corresponding ODEs are:

$$\dot{z}_T = \alpha_z(z_T^{tot} - z_T)z_A - \delta_z z_T x_{R,I}, \tag{27}$$
$$\dot{z}_A = \kappa_z(z_A^{tot} - z_A - z_T) - \alpha_z(z_T^{tot} - z_T)z_A - \boxed{\nu_z x_{R,I} z_A}, \tag{28}$$
$$\dot{x}_{R,I} = \beta_x x_T - \delta_z z_T x_{R,I} - \phi_x x_{R,I} - \boxed{\nu_z x_{R,I} z_A}, \tag{29}$$
$$\dot{x}_T = \alpha_x(x_T^{tot} - x_T)x_A - \delta_x x_T z_{R,I}, \tag{30}$$
$$\dot{x}_A = \kappa_x(x_A^{tot} - x_A - x_T) - \alpha_x(x_T^{tot} - x_T)x_A - \boxed{\nu_x x_A z_{R,I}}, \tag{31}$$
$$\dot{z}_{R,I} = \beta_z z_T - \delta_x x_T z_{R,I} - \phi_z z_{R,I} - \boxed{\nu_x x_A z_{R,I}}. \tag{32}$$

Boxes highlight the terms corresponding to titration reactions. The two modules correspond to the subsystems z_T–z_A–$x_{R,I}$ and x_T–x_A–$z_{R,I}$.

Proposition 17. *The solutions of the two separated modules, as well as those of the interconnected system* (27)–(32)*, are globally bounded.*

Proof. Analogous to the proofs of Propositions 5, 11 and 13. □

3.1 Analysis in the absence of direct titration reactions

3.1.1 Equilibrium conditions

We consider system (27)–(32) in the absence of titration reactions, *i.e.*, with $\nu_x = \nu_z = 0$. We begin by setting equations $\dot{x}_T + \dot{x}_A = 0$ and we combine them with $\dot{x}_T = 0$. From here $\bar{z}_{R,I}$ is isolated as:

$$\bar{z}_{R,I} = \frac{\alpha_x(x_T^{tot} - \bar{x}_T)\,\bar{x}_A}{\delta_x \bar{x}_T} = \frac{\kappa_x(x_A^{tot} - \bar{x}_A - \bar{x}_T)}{\delta_x \bar{x}_T}.$$

We then find a relationship between $\bar{x}_T$ and $\bar{x}_A$ at steady state:

$$\bar{x}_A = \frac{\kappa_x(x_A^{tot} - \bar{x}_T)}{\alpha_x(x_T^{tot} - \bar{x}_T) + \kappa_x}.$$

Finally, setting equations $\dot{x}_T + \dot{x}_A = 0$ and $\dot{z}_{R,I} = 0$ we get:

$$\bar{z}_T = \frac{\kappa_x}{\beta_z}(x_A^{tot} - \bar{x}_A(\bar{x}_T) - \bar{x}_T) + \frac{\phi_z}{\beta_z}\bar{z}_{R,I}(\bar{x}_T). \tag{33}$$

The system is symmetric, so we now use the same procedure to obtain $\bar{x}_T(\bar{z}_T)$. We start setting $\dot{z}_T + \dot{z}_A = 0$ and equation $\dot{x}_{R,I} = 0$. We find:

$$\bar{x}_T = \frac{\kappa_z}{\beta_x}(z_A^{tot} - \bar{z}_A(\bar{z}_T) - \bar{z}_T) + \frac{\phi_x}{\beta_x}\bar{x}_{R,I}(\bar{z}_T). \tag{34}$$

Both equilibrium conditions are monotonically decreasing, which guarantees uniqueness of the equilibrium.

3.1.2 Structural bistability

The Jacobian matrix of system (27)–(32) with $\nu_x = \nu_z = 0$ is:

$$J_\nu = \begin{bmatrix} -\alpha_z\bar{z}_A - \delta_z\bar{x}_{R,I} & \alpha_z(z_T^{tot} - \bar{z}_T) & \delta_z\bar{z}_T & 0 & 0 & 0 \\ -\kappa_z + \alpha_z\bar{z}_A & -\kappa_z - \alpha_z(z_T^{tot} - \bar{z}_T) & 0 & 0 & 0 & 0 \\ \delta_z\bar{x}_{R,I} & 0 & -\delta_z\bar{z}_T - \phi_x & -\beta_x & 0 & 0 \\ 0 & 0 & 0 & -\alpha_x\bar{x}_A - \delta_x\bar{z}_{R,I} & \alpha_x(x_T^{tot} - \bar{x}_T) & \delta_x\bar{x}_T \\ 0 & 0 & 0 & -\kappa_x + \alpha_x\bar{x}_A & -\kappa_x - \alpha_x(x_T^{tot} - \bar{x}_T) & 0 \\ -\beta_z & 0 & 0 & \delta_x\bar{z}_{R,I} & 0 & -\delta_x\bar{x}_T - \phi_z \end{bmatrix} \tag{35}$$

Here, the sign of the third and the sixth rows and columns has been changed (corresponding to a sign change for variables $x_{R,I}$ and $z_{R,I}$).

We say that a system is a *strong candidate bistable system* [1, 2] if it can become unstable exclusively due to a real eigenvalue that becomes positive.

Proposition 18. *Under Assumptions 1, 2, 3, and 4, system* (27)–(32) *is a strong candidate bistable system.*

Proof. We remind that, under our assumptions, $-\kappa_z + \alpha_z z_A > 0$ and $-\kappa_x + \alpha_x x_A > 0$. Then a similarity transformation $\hat{J}_\nu = T^{-1}J_\nu T$ can be applied, with $T = \text{diag}\{-1,\ -1,\ -1,\ 1,\ 1,\ 1\}$, such that $\hat{J}_\nu$ has non–negative off–diagonal entries, namely is a Metzler matrix, and negative diagonal entries. It is known (see for instance [3]) that a Metzler matrix has a real dominant eigenvalue: in this case, this means that an eigenvalue λ_1 exists such that $Re(\lambda_i) \leq \lambda_1$, for $i = 2, 3, \dots, 5$. Hence the proof follows. $\square$

The bistable nature of this system can be explained as follows. The transition to instability, if it happens, is due to a real eigenvalue which crosses the origin (0), becoming positive. This implies that the determinant of the matrix changes sign. Being the overall solution bounded, this implies that other two equilibria, both locally stable, necessarily appear (see [1, 2] for details).

3.2 Analysis in the presence of direct titration reactions

3.2.1 Equilibrium conditions

To derive $\bar{z}_T$ as a function of $\bar{x}_T$, we set equations $\dot{x}_T + \dot{x}_A = 0$ and $\dot{x}_T = 0$, and we find two different expressions for $\bar{z}_{R,I}$. Equating these expressions we obtain:

$$\bar{z}_{R,I} = \frac{\alpha_x(x_T^{tot} - \bar{x}_T)\,\bar{x}_A}{\delta_x\bar{x}_T} = \frac{\kappa_x(x_A^{tot} - \bar{x}_A - \bar{x}_T)}{\delta_x\bar{x}_T + \nu_x\bar{x}_A},$$

and we find a relationship between $\bar{x}_T$ and $\bar{x}_A$ at steady state. As done before, we derive the equilibrium of $\bar{x}_A$ as the solution of the second order equation $a_x\bar{x}_A^2+b_x\bar{x}_A+c_x$, where $a_x = \left(\frac{\alpha_x\nu_x}{\delta_x}\right)\frac{x_T^{tot}-\bar{x}_T}{\bar{x}_T}$, $b_x = \left(\alpha_x(x_T^{tot}-\bar{x}_T)+\kappa_x\right)$ and $c_x = -\kappa_x(x_A^{tot}-\bar{x}_T)$. Assuming $x_A^{tot} > x_T^{tot}$, since $a_xc_x < 0$, the only admissible positive solution is:

$$\bar{x}_A(\bar{x}_T) = \frac{-b_x+\sqrt{b_x^2-4a_xc_x}}{2a_x}.$$

Finally, setting equations $\dot{x}_T+\dot{x}_A = 0$ and $\dot{z}_{R,I} = 0$ we get:

$$\bar{z}_T = \frac{\kappa_x}{\beta_z}(x_A^{tot}-\bar{x}_A(\bar{x}_T)-\bar{x}_T)+\frac{\phi_z}{\beta_z}\bar{z}_{R,I}(\bar{x}_T). \tag{36}$$

With a similar procedure we can get the equilibrium condition for $\bar{x}_T(\bar{z}_T)$ and the remaining equilibria. Once we find the admissible equilibrium values $\bar{z}_T$, $\bar{z}_A$, $\bar{x}_T$ and $\bar{x}_A$ we can find $\bar{z}_{R,I}$ and $\bar{x}_{R,I}$.

$$\begin{aligned}
\dot{x}_{R,I} = 0 \quad &\implies \quad \bar{z}_{R,I} = \frac{\kappa_x(x_A^{tot}-\bar{x}_A-\bar{x}_T)}{\delta_x\bar{x}_T+\nu_x\bar{x}_A},\\
\dot{z}_{R,I} = 0 \quad &\implies \quad \bar{x}_{R,I} = \frac{\kappa_z(z_A^{tot}-\bar{z}_A-\bar{z}_T)}{\delta_z\bar{z}_T+\nu_z\bar{z}_A}.
\end{aligned}$$

3.2.2 Structural bistability

In the presence of direct titration reactions the Jacobian becomes:

$$J_\nu = \tag{37}$$
$$\begin{bmatrix}
-\alpha_z\bar{z}_A-\delta_z\bar{x}_{R,I} & \alpha_z(z_T^{tot}-\bar{z}_T) & \delta_z\bar{z}_T & 0 & 0 & 0\\
-\kappa_z+\alpha_z\bar{z}_A & -\kappa_z-\alpha_z(z_T^{tot}-\bar{z}_T)-\nu_z\bar{x}_{R,I} & \nu_z\bar{z}_A & 0 & 0 & 0\\
\delta_z\bar{x}_{R,I} & \nu_z\bar{x}_{R,I} & -\delta_z\bar{z}_T-\nu_z\bar{z}_A-\phi_x & -\beta_x & 0 & 0\\
0 & 0 & 0 & -\alpha_x\bar{x}_A-\delta_x\bar{z}_{R,I} & \alpha_x(x_T^{tot}-\bar{x}_T) & \delta_x\bar{x}_T\\
0 & 0 & 0 & -\kappa_x+\alpha_x\bar{x}_A & -\kappa_x-\alpha_x(x_T^{tot}-\bar{x}_T)-\nu_x\bar{z}_{R,I} & \nu_x\bar{x}_A\\
-\beta_z & 0 & 0 & \delta_x\bar{z}_{R,I} & \nu_x\bar{z}_{R,I} & -\delta_x\bar{x}_T-\nu_x\bar{x}_A-\phi_z
\end{bmatrix}$$

As done earlier, the sign of the third and the sixth rows and columns has been changed (corresponding to a sign change for variables $x_{R,I}$ and $z_{R,I}$).

In the presence of direct titration reactions it is more difficult to formally show that the feedback of the two subsystems is a candidate bistable system. We can however note that:
a) For "small" ν_x and ν_z, the two subsystems are "almost" in the same condition of Proposition 18, hence bistability occurs. Also, if we can assume that $\bar{z}_A > \kappa_z/\alpha_z$ and $\bar{x}_A > \kappa_x/\alpha_x$, then the same considerations as in the case of no titration apply and a bistable behavior is expected.
b) More than one equilibrium point may appear for suitable choice of the parameters. When three equilibria appear, if the Jacobian is invertible, then necessarily one of the equilibria is unstable with a real positive unstable eigenvalues; this can be explained with the so called degree theory; we refer the reader to reference [1] for additional details. The other two equilibria are expected to be stable.
c) Numerical simulations on a wide range of parameters confirm that this system can be bistable.

3.3 Numerical simulations

3.3.1 Probability of bistable behavior

As done for the oscillator, we explored the probability of obtaining a bistable behavior for random choices of the parameters around the nominal set in Table S2. The reaction rate parameters were randomly selected in the range from 10^{-2} to 10^{2} their nominal value; parameters z_T^{tot}, x_A^{tot}, x_T^{tot}, x_A^{tot} were instead varied between one tenth and ten times their nominal value. A set of parameters is classified as bistable if a) three equilibria are identified by numerically finding the intersections of the equilibrium conditions derived earlier, and b) two of these equilibria are stable (all eigenvalues have negative real part) and one is unstable (at least one eigenvalue has positive real part). Fig. S7 and S8 show that the system can exhibit bistability in a wide range of parameters, however large titration reaction rates (Fig. S8) significantly increase the probability of bistable behavior.

Figure S7: Absence of direct titration reactions. Log plot showing the correlation between randomly chosen parameters that yield bistable behavior. Nominal parameters (Table S2) are shown in the orange diamond; reaction rate parameters were varied between a factor 10^{-2} and 10^{2} of their nominal value (with $\nu = 0$), while total concentrations were varied between one tenth and ten times their nominal value.

3.3.2 Bistable behavior in a region of the parameter space

We use parameters listed in Table S2 to explore numerically the bistability regions. As done for the randomized parameter classification, a parameter set yields a bistable behavior if three equilibria, two stable and one unstable,

Figure S8: Presence of direct titration reactions. Log plot showing the correlation between randomly chosen parameters that yield bistable behavior. Nominal parameters (Table S2) are shown in the orange diamond; parameters were varied between a factor 10^{-2} and 10^{2} of their nominal value.

Table S2: Nominal parameters for the bistable circuit

Units: $[\mathrm{nM}]$	Units: $[1/\mathrm{s}]$	Units: $[1/\mathrm{M/s}]$
$z_T^{tot} = 100$	$\beta_z = 0.0021$	$\alpha_z = 3 \times 10^4$
$x_T^{tot} = 100$	$\beta_x = \beta_z$	$\alpha_x = \alpha_z$
$z_A^{tot} = 200$	$\kappa_z = 3 \times 10^{-4}$	$\delta_z = 3 \times 10^4$
$x_A^{tot} = 200$	$\kappa_x = \kappa_z$	$\delta_x = \delta_z$
	$\phi_z = 0.001$	$\nu_z = \delta_z$
	$\phi_x = \phi_z$	$\nu_x = \nu_z$

are identified. Here, we vary only two parameters at a time, keeping the others fixed at their nominal value. In Fig. S9 and S10 we show the bistability domains (orange regions), in the absence of direct titration reactions (Fig. S9) and in the presence of titration (Fig. S10).

In the absence of titration reactions there are many pairs of parameters where the bistability region is very narrow. This makes the system less robust over the parameter space since any change in the parameters will cause the system to lose bistability. It also shows that there is a linear correlation in many pairs of parameters for a bistable behavior: (β_z, β_x), (κ_z, κ_x), (κ_x, z_A^{tot}), (β_z, x_A^{tot}), (κ_z, x_A^{tot}), (δ_x,ϕ_z) and (δ_z, ϕ_x) show a positive correlation, while (β_z, κ_z), (β_z, z_A^{tot}), (κ_z, z_A^{tot}), (β_x, x_A^{tot}) and (κ_x, x_A^{tot}) show a negative correlation to present bistable behavior. Fig. S10 clearly shows that all the regions of bistability are expanded when the titration reaction are present.

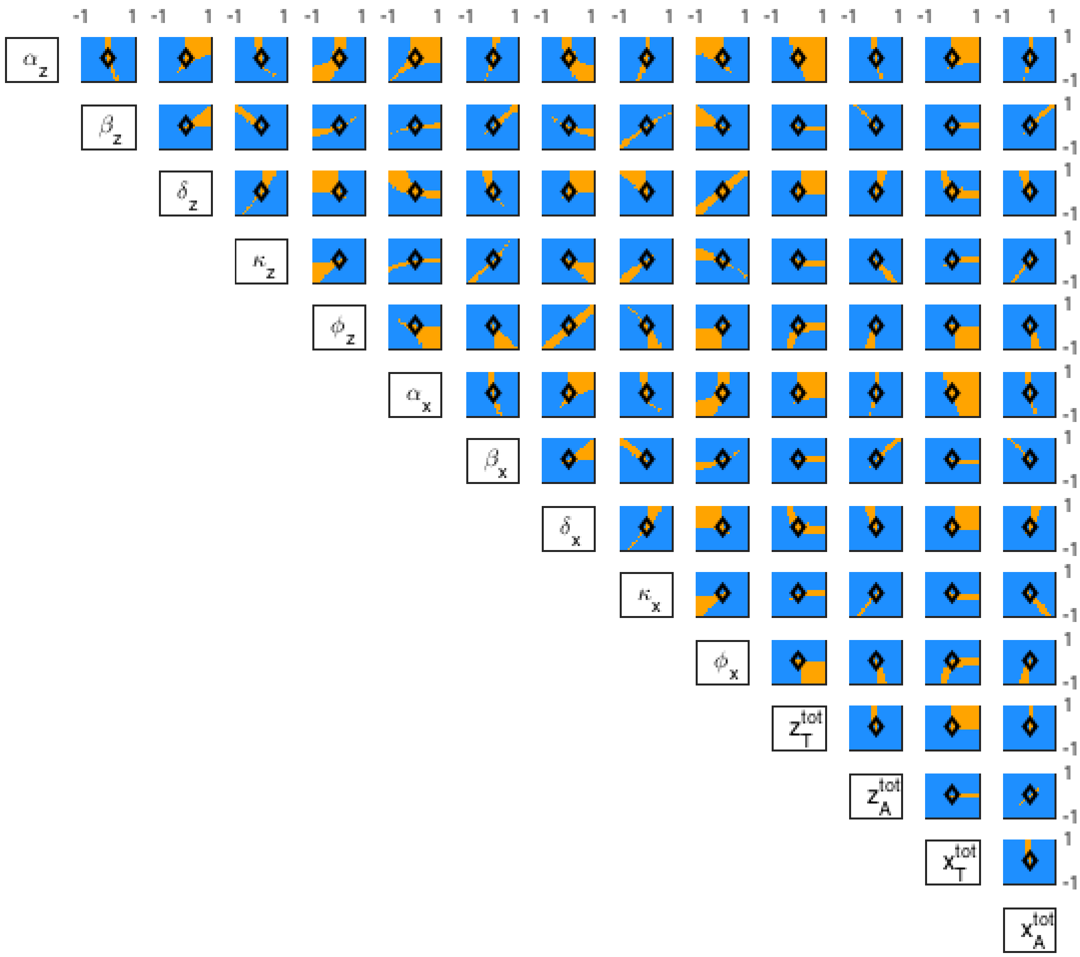

Figure S9: Absence of direct titration reactions. Axes are in log scale. The orange areas are bistable regions. Blue areas correspond to a unique stable steady state. Nominal parameters are shown as a black diamond.

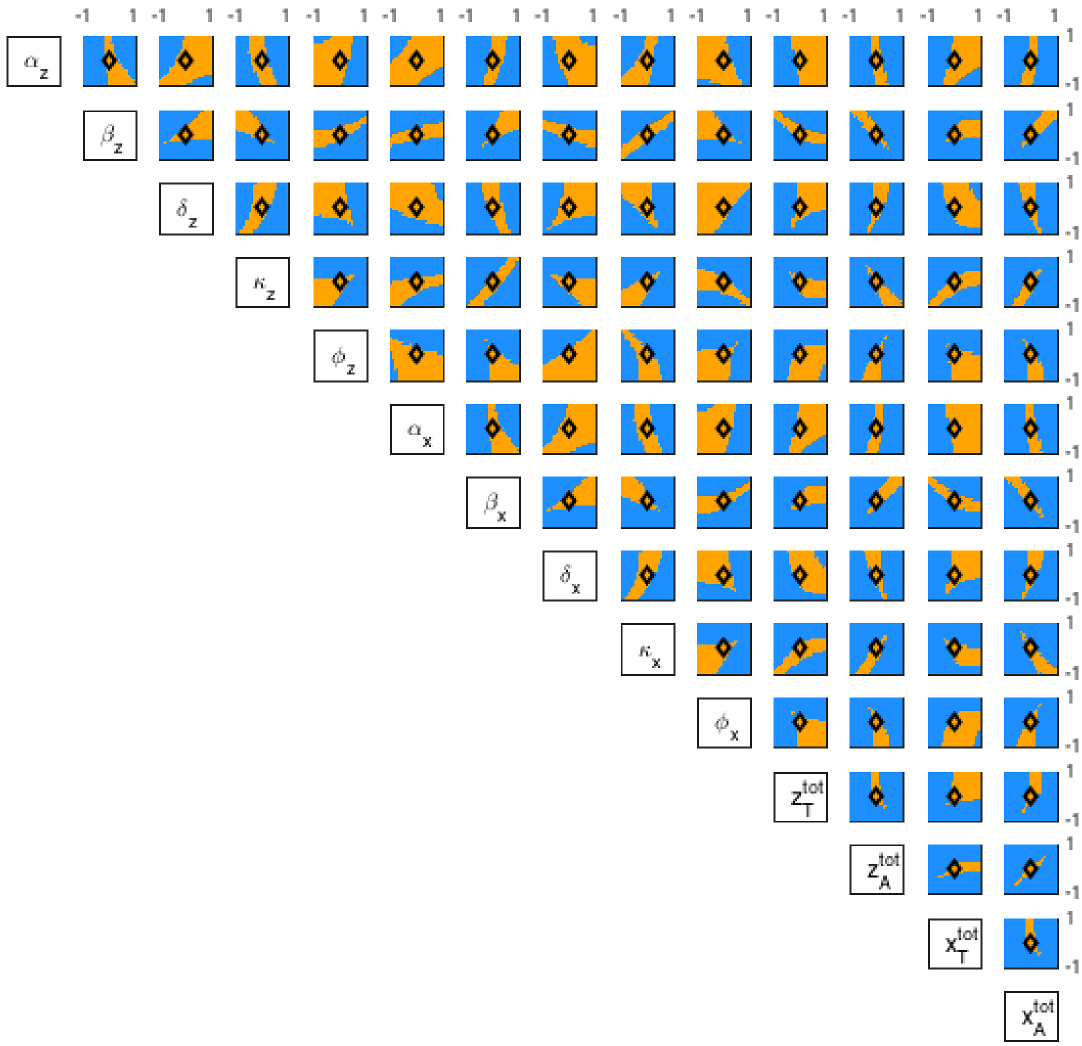

Figure S10: Presence of direct titration reactions. Axes are in log scale. The orange areas are bistable regions, which are clearly expanded relative to Fig. S9. Blue areas correspond to a unique stable steady state. Nominal parameters are shown as a black diamond

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