# Threshold Responses to Morphogen Gradients by Zero-Order Ultrasensitivity

# Supplementary Material

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# 1. Scheme of Yan Degradation Network

We analyzed the circuitry targeting Yan for degradation using mathematical modeling. We consider the number of binding events as a free parameter, n. We also assumed that Yan phosphorylation is reversible, and that dephosphorylation is catalyzed by some phosphatase P (Fig. S1). To account for the possibility of an additional positive feedback branch, we extended the model, by allowing an arbitrary dependence of the rate constants on Yan, or Yan-p levels. As a specific feedback mechanism, we assumed that Yan dephosphorylation rate decreases monotonously with Yan (Fig. S1).

The input to this model is the level of active MAPK, while its output is the total level of Yan, $Y^{tot} = Y + Y_p$. This last assumption reflects the capacity of both phosphorylated and dephosphorylated Yan to repress target-gene expression. Since activated MAPK is spatially graded, tight degradation borders are achieved when the $Y^{tot}$ displays a switch-like dependency on the level of activated MAPK.

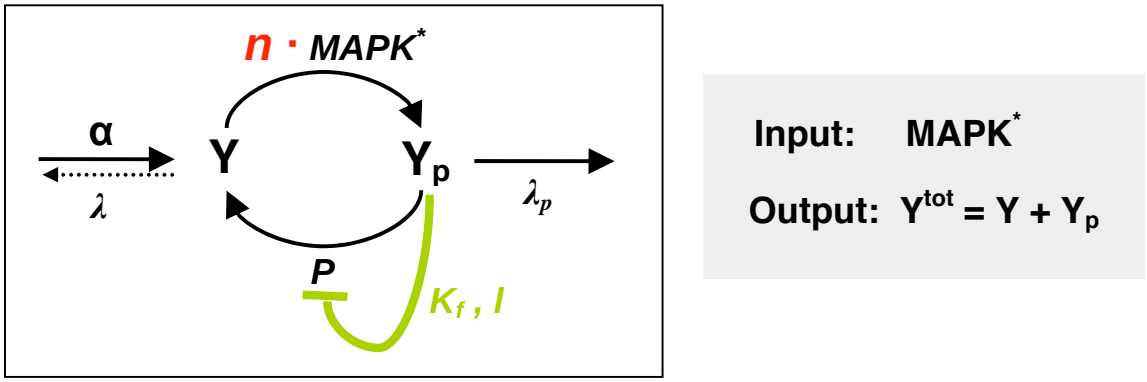
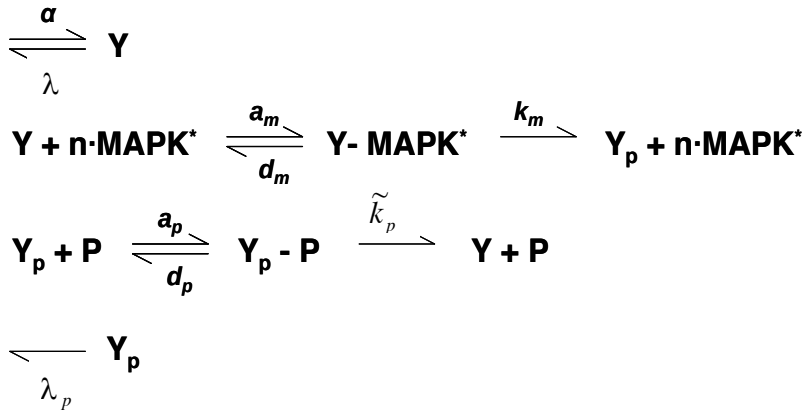



**Figure S1.** Models of Yan degradation network

The behavior of the system was examined at four different limits. First, the non-cooperative linear model was considered, where we assume that a single phosphorylation of Yan by MAPK is required (n = 1), and that the corresponding enzymatic reactions are first-order (i.e. enzymes are in excess and bind most of the Yan molecules). Second, the cooperative, linear model with n > 1 was examined. Third, we consider the non-cooperative zero-order case, with n=1 and reactions that function at the zero-order regime. In this case enzymes are present at limiting amounts, most Yan is free and $Y^{tot} >> K_m, K_p$. Finally, we considered a feedback system with a single phosphorylation site (n = 1) and enzymatic reactions that function at first-order.

## 2. Yan Degradation Network Equations

Yan degradation network kinetics is based on the following reactions:




The reactions result in the following equations:

2.1. $$\frac{dY}{dt} = -a_m Y[MAPK_f^*]^n + d_m[YMAPK^*] + \tilde{k}_p(Y_p)\cdot[Y_p P] + \alpha - \lambda Y$$

2.2. $$\frac{d[YMAPK^*]}{dt} = a_m Y[MAPK_f^*]^n - (d_m + k_m)\cdot[YMAPK^*]$$

2.3. $$\frac{dY_p}{dt} = -a_p Y_p[P_f] + d_p[Y_p P] + k_m[YMAPK^*] - \lambda_p Y_p$$

2.4. $$\frac{d[Y_p P]}{dt} = a_p Y_p[P_f] - (d_p + \tilde{k}_p(Y_p))\cdot[Y_p P]$$

, and two enzyme conservation equations:

2.5. $$[MAPK^*] = n\cdot[YMAPK^*] + [MAPK_f^*]$$

2.6. $$[P] = [Y_p P] + [P_f]$$

, where $\tilde{k}_p(Y_p) = k_p \frac{K_f^l}{K_f^l + Y_p^l}$ consists of a possible positive feedback branch.

Here $[MAPK_f^*]$ and $[P_f]$ denote the concentrations of free activated MAPK and the free phosphatase P, respectively. $[YMAPK_f^*]$ and $[Y_p P]$ denote the concentration of active MAPK and the phosphatase at their complex form. The dissociation constants are defined as:

2.7. $$(K_m)^n \equiv \frac{d_m + k_m}{a_m} \quad ; \quad K_p \equiv \frac{d_p + k_p}{a_p}$$

The equations were solved numerically and analytically at four different limits: non-cooperative first-order kinetics, cooperative first-order kinetics, non-cooperative first-order with positive feedback, and non-cooperative zero-order kinetics.

# 3. Steady-State Solution of First-Order Networks

At first-order Yan level is limiting with respect to both enzyme levels, and both dissociation constants: $Y^{tot} << [MAPK^*], [P]$ ; $Y^{tot} << K_m, K_p$. In this section we will discuss the steady state solution of both the non-cooperative and cooperative cases.

## 3.a. Non-cooperative first order networks

For the non-cooperative case (n = 1) the quasi-steady-state equations reduce to:

3.1. $$\frac{dY}{dt} = -\frac{k_m}{K_m}[MAPK^*]\cdot Y + \frac{k_p}{K_p}[P]\cdot Y_p + \alpha - \lambda Y$$

3.2. $$\frac{dY_p}{dt} = \frac{k_m}{K_m}[MAPK^*]\cdot Y - \frac{k_p}{K_p}[P]\cdot Y_p - \lambda_p Y_p$$

and the steady-state solution at the non-cooperative first-order case is:

3.3. $$Y = \frac{\alpha/\lambda}{1+\frac{[MAPK^*]}{C}} \quad ; \quad Y_p = \frac{\alpha/\lambda_p}{1+\frac{C}{[MAPK^*]}} \quad ; \quad C \equiv K_m \cdot \frac{\lambda\cdot\left(1+\frac{k_p[P]}{K_p\lambda_p}\right)}{k_m}$$

The resulting Y and $Y_p$ levels are smooth functions of the activated MAPK level, which indicates that the network's output: $Y^{tot} = Y + Y_p$ is not sensitive to the network's input (total activated MAPK level). The same gradual response was observed in the simulations (Figs. 3, S2, S3A).

As expected, at very low activated MAPK levels ($[MAPK^*] << C$) all Yan is non-phosphorylated and its level is maximal ($\alpha/\lambda$), whereas at very high MAPK levels ($[MAPK^*] >> C$) all Yan is phosphorylated and its level is minimal ($\alpha/\lambda_p$ ; $\lambda_p >> \lambda$).

## 3.b. Cooperative first order networks

Using the same first-order assumptions for the cooperative case (n > 1), the following quasi steady-state equations are reached:

3.4. $$\frac{dY}{dt} = -a_m Y[MAPK_f^*]^n + d_m[YMAPK^*] + \frac{k_p}{K_p}[P]\cdot Y_p + \alpha - \lambda Y$$

3.5. $$\frac{dY_p}{dt} = -\frac{k_p}{K_p}[P]\cdot Y_p + k_m[YMAPK^*] - \lambda_p Y_p$$

3.6. $$[YMAPK^*] = \frac{Y[MAPK_f^*]^n}{(K_m)^n} \quad ; \quad [MAPK^*] = n\cdot[YMAPK^*] + [MAPK_f^*]$$

For simplicity, we assumed that most of the activated MAPK is free, and therefore, equation 3.6 can be rewritten as:

3.7. $$[YMAPK^*] = Y \cdot \frac{[MAPK^*]^n}{(K_m)^n}$$ (See chapter 9 for explicit derivation)

Substituting equation (3.7) into equations (3.4) and (3.5) will result in:

3.8. $$\frac{dY}{dt} = -\frac{k_m}{(K_m)^n}[MAPK^*]^n \cdot Y + \frac{k_p}{K_p}[P] \cdot Y_p + \alpha - \lambda Y$$

3.9. $$\frac{dY_p}{dt} = \frac{k_m}{(K_m)^n}[MAPK^*]^n \cdot Y - \frac{k_p}{K_p}[P] \cdot Y_p - \lambda_p Y_p$$

The steady-state solution at cooperative first-order kinetics is:

3.10. $$Y = \frac{\alpha/\lambda}{1 + \frac{[MAPK^*]^n}{C_n}} \quad ; \quad Y_p = \frac{\alpha/\lambda_p}{1 + \frac{C_n}{[MAPK^*]^n}} \quad ; \quad C_n \equiv (K_m)^n \frac{\lambda \cdot \left(1 + \frac{k_p[P]}{K_p \lambda_p}\right)}{k_m}$$

In contrast to the non-cooperative case, Y and $Y_p$ levels are sensitive to the total activated MAPK level, which indicates that the output: $Y^{tot} = Y + Y_p$ is sensitive to the input, as seen in the simulations (Figs. 3, S2, S3B).

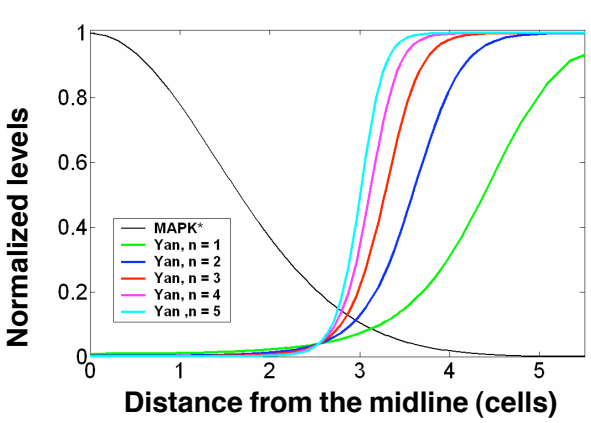



**Figure S2.** Cooperativity at first-order kinetics generates a sharp threshold of Yan degradation.

Yan steady state concentration was found by numerical simulations (see Methods for parameters used). The concentration levels are normalized with the corresponding maximal concentrations. The colored curves designate Yan levels at steady state, where each graph corresponds to a different Hill coefficient (n). As expected, Yan degradation network generates a sharper switch as the cooperativity is increased (large n).

## 4. Steady-State Solution of Zero-Order Networks

At zero-order Yan level is in excess with respect to the two enzyme levels, and both dissociation constants: $Y^{tot} >> [MAPK^*],[P]$ ; $Y^{tot} >> K_m, K_p$. When $Y, Y_p = O(Y^{tot})$, the quasi-steady-state equations are:

4.1. $$\frac{dY}{dt} = -k_m[MAPK^*] + k_p[P] + \alpha - \lambda Y$$

4.2. $$\frac{dY_p}{dt} = k_m[MAPK^*] - k_p[P] - \lambda_p Y_p$$

The steady-state solution of these equations is:

4.3. $$Y = \frac{\alpha - k_m[MAPK^*] + k_p[P]}{\lambda} \quad ; \quad Y_p = \frac{k_m[MAPK^*] - k_p[P]}{\lambda_p}$$

At the classical zero-order model (Fig. 7A) the switch-like response is generated by a single threshold (defined at: $k_1[E_1] = k_2[E_2]$). However, in the Yan degradation network (Fig. 7B) a switch is generated by a two threshold mechanism. A decrease in total activated MAPK level will result in a decrease in the phophorylated Yan level and a corresponding increase in its non-phophorylated form. Since $Y \le Y^{\max} = \alpha/\lambda$ and $Y_p \ge 0$, the steady-state solution is valid only if: $[MAPK^*] \ge \frac{k_p[P]}{k_m} \equiv T_1$. If the activated MAPK level is lower than this threshold ($T_1$), all Yan is non-phophorylated and the total Yan level reaches its maximal value ($\alpha/\lambda$).

When activated MAPK level increases, the level of phophorylated Yan is increased while the level of its non-phophorylated form decreases. Since $Y_p \le Y_p^{\max} = \alpha/\lambda_p$ and $Y \ge 0$, the steady-state solution is valid only if: $[MAPK^*] \le \frac{\alpha + k_p[P]}{k_m} \equiv T_2$. If the total activated MAPK level is higher than this threshold ($T_2$), all Yan is phophorylated and the total Yan level reaches its minimal value ($\alpha/\lambda_p$).

The two threshold mechanisms can be summarized as follows:

4.4. $$Y^{tot} = \begin{cases} Y^{\max} = \frac{\alpha}{\lambda} & , [MAPK^*] < T_1 \\ linear\ in\ [MAPK^*] & , T_1 < [MAPK^*] < T_2 \\ Y_p^{\max} = \frac{\alpha}{\lambda_p} & , [MAPK^*] > T_2 \end{cases}$$

There are two features which are important for generating a switch-like response of Yan degradation network at zero-order kinetics. First, Yan degradation rate is much higher in its

phophorylated form ($\lambda << \lambda_p$), and therefore, $Y^{\max} >> Y_p^{\max}$. This property assures that the system's output has a large range, and therefore, a switch can potentially be generated if a transition in the order of magnitude of this range will occur within a small change in input values. Second, protein production rates are typically much slower than protein dephosphorylation reactions, such that: $\alpha << k_p[P]$, and therefore, $T_2 - T_1 << T_1$. This property assures that a large transition in the system's output will occur within a small input range, and a switch will be generated. Note that since $\alpha << k_p[P]$, the switch does not change its position upon over-expression (increasing α).

Numerical simulations of Yan degradation network at zero-order kinetics also exhibit a switch-like response, and clearly agree with the analytical expectations (Figs. 3, S3D).

# 5. The Effect of Positive Feedback in Covalent Modification Systems

As we discussed in the main text, our system is related to the classical zero-order model (Fig. 7A). To illustrate the effect of a positive feedback branch on the level of Yan, we solved the simple case of the classical covalent modification model. In section 5.a. we will formulate the first order network with a general feedback branch, and show its steady-state solution. In section 5.b. we will prove that any first order network, with or without a feedback branch, is sensitive to inhibition and over-expression of the substrate. An example of a network with a positive feedback will be introduced at section 5.c.

## 5.a. First order networks with a general feedback branch

Considering the enzymes $E_1$, and $E_2$ as activated MAPK and phosphatase, respectively, the dynamic equations of the non-cooperative classical model *without feedback* are:

5.1. $$\frac{dY}{dt} = -a_m Y[MAPK_f^*] + d_m[YMAPK^*] + k_p[Y_p P]$$

5.2. $$\frac{d[YMAPK^*]}{dt} = a_m Y[MAPK_f^*] - (d_m + k_m)\cdot[YMAPK^*]$$

5.3. $$\frac{dY_p}{dt} = -a_p Y_p[P_f] + d_p[Y_p P] + k_m[YMAPK^*]$$

5.4. $$\frac{d[Y_p P]}{dt} = a_p Y_p[P_f] - (d_p + k_p)\cdot[Y_p P]$$

At quasi steady-state these equations reduce to:

5.5. $$\frac{dY}{dt} = -k_m[MAPK^*]\cdot\frac{Y}{Y + K_m} + k_p[P]\cdot\frac{Y_p}{Y_p + K_p}$$

5.6. $$\frac{dY_p}{dt} = k_m[MAPK^*]\cdot\frac{Y}{Y + K_m} - k_p[P]\cdot\frac{Y_p}{Y_p + K_p}$$

At first-order kinetics ($Y^{tot} << K_m, K_p$), equations 5.5 and 5.6 can be rewritten as:

5.7. $$\frac{dY}{dt} = -\frac{k_m}{K_m}[MAPK^*]\cdot Y + \frac{k_p}{K_p}[P]\cdot Y_p \quad ; \quad \frac{dY_p}{dt} = \frac{k_m}{K_m}[MAPK^*]\cdot Y - \frac{k_p}{K_p}[P]\cdot Y_p$$

Put another way:

5.8. $$\frac{dY_p}{dt} = C_m[MAPK^*]\cdot Y - C_p\cdot Y_p \qquad \text{, where } \; C_m \equiv \frac{k_m}{K_m} \quad ; \quad C_p \equiv \frac{k_p}{K_p}[P]$$

,and the equation can be solved completely using the conservation equation:

5.9. $$Y^{tot} = Y + Y_p + \Delta \quad ; \quad \Delta \equiv [YMAPK^*] + [Y_p P]$$

Note that at quasi steady-state: $\Delta = \frac{Y \cdot [MAPK_f^*]}{K_m} + \frac{Y_p \{P_f]}{K_p}$ , and at first order:

$\Delta = \frac{Y \cdot [MAPK^*]}{K_m} + \frac{Y_p \{P]}{K_p}$.

The steady-state solution of equations 5.8 and 5.9 is:

5.10. $$Y_p = \frac{F \cdot [MAPK^*]}{F \cdot [MAPK^*] + 1} \cdot \left(Y^{tot} - \Delta\right) \quad \text{, where: } F \equiv \frac{C_m}{C_p}.$$

F is a positive constant which depends only on rate constants and phosphatase concentration, and does not depend on the total Yan concentration.

When a feedback branch is added to the network, the coefficient $C_m$ and/or $C_p$ depends on the network output, i.e. $C_m = C_m(Y_p)$ and/or $C = C_p(Y_p)$. Therefore, the coefficient F is no longer constant but depends on the output $Y_p$. In case of a positive feedback, $C_m$ is monotonically increasing with $Y_p$ and/or $C_p$ is monotonically decreasing with $Y_p$, and therefore, $F(Y_p)$ will increase monotonically with $Y_p$. On the other hand, in the case of a negative feedback F will decrease monotonically with $Y_p$.
Note that $F(Y_p)$ depends on $Y^{tot}$ only through $Y_p$.

The general steady-state solution for a first order covalent modification network with a feedback branch can be written as:

5.11. $$Y_p = \frac{F(Y_p) \cdot [MAPK^*]}{F(Y_p) \cdot [MAPK^*] + 1} \cdot \left(Y^{tot} - \Delta\right)$$

## 5.b. Sensitivity of non-cooperative first-order networks steady-states to over expression.

Our experimental results show that the border of Yan degradation does not change its position upon over-expression, i.e. the input signal needed for generating a given output is not changed when the substrate concentration is increased. In this section we will show that *non-cooperative first-order* covalent modification networks are sensitive to changes in substrate concentration, whether the network has a feedback loop or not. The formulation of section 5.a. will be used.

**Claim:**

Upon over expression by a factor of $\beta > 1$ ($Y^{tot} \rightarrow \beta \cdot Y^{tot}$), the input signal will have to decrease *at least* by a factor $\beta$ ($[MAPK^*] \rightarrow [MAPK^*] / \beta$) in order for the output to stay unchanged ($Y_p \rightarrow Y_p$).

In the case of inhibition ($\beta < 1$ ; $Y^{tot} \rightarrow \beta \cdot Y^{tot}$), the input signal will have to increase *at least* by a factor $1/\beta$ ($[MAPK^*] \rightarrow [MAPK^*] / \beta$) in order for the output to stay unchanged ($Y_p \rightarrow Y_p$).

**Proof:**

Notations: $M = [MAPK^*]$ = input.

$\overline{X}$ = The value of the variable X after the over-expression / inhibition.

Consider a change $Y^{tot} \rightarrow \beta \cdot Y^{tot}$, for which $Y_p = \overline{Y}_p$. What is the new input ($\overline{M}$)?

Using 5.11 $Y_p$ for $\overline{Y}_p$, we get:

5.12. $$Y_p = \frac{F(Y_p) \cdot M}{F(Y_p) \cdot M + 1} \cdot \left(Y^{tot} - \Delta\right)$$

5.13. $$\overline{Y}_p = \frac{F(\overline{Y}_p) \cdot \overline{M}}{F(\overline{Y}_p) \cdot \overline{M} + 1} \cdot \left(\beta \cdot Y^{tot} - \overline{\Delta}\right)$$

Since $Y_p = \overline{Y}_p$, we can equate 5.12 and 5.13, and use $F(Y_p) = F(\overline{Y}_p)$:

5.14. $$Y_p = \frac{F(Y_p) \cdot M}{F(Y_p) \cdot M + 1} \cdot \left(Y^{tot} - \Delta\right) = \frac{F(Y_p) \cdot \overline{M}}{F(Y_p) \cdot \overline{M} + 1} \cdot \left(\beta \cdot Y^{tot} - \overline{\Delta}\right)$$

Designating $X \equiv \left(F(Y_p) \cdot M\right)^{-1}$ ; $\overline{X} \equiv \left(F(Y_p) \cdot \overline{M}\right)^{-1}$, 5.14 can be rewritten as:

5.15. $$\frac{Y^{tot} - \Delta}{1 + X} = \frac{\beta \cdot Y^{tot} - \overline{\Delta}}{1 + \overline{X}}$$ , put another way:

5.16. $$\overline{X} = \beta \cdot X \cdot A + B \quad ; \qquad A \equiv \frac{Y^{tot} - \overline{\Delta} \big/ \beta}{Y^{tot} - \Delta} \quad ; \quad B \equiv \frac{Y^{tot}(\beta - 1) - (\overline{\Delta} - \Delta)}{Y^{tot} - \Delta}$$

Using the conservation equation: $Y^{tot} = Y + Y_p + \Delta = Y \cdot \left(1 + \frac{M}{K_m}\right) + Y_p \cdot \left(1 + \frac{[P]}{K_p}\right)$, and since $Y^{tot} \rightarrow \beta \cdot Y^{tot}$ without changing $Y_p$, it is clear that Y is changed by a factor larger than $\beta$. The complexes: $\Delta = \frac{Y \cdot [MAPK^*]}{K_m} + \frac{Y_p \cdot [P]}{K_p}$, include two terms: The first one, which changes by a

factor larger than β, and the second one that doesn't change at all. In the following derivation I will assume that $\overline{\Delta} \cong \beta \cdot \Delta$. In the case of β>1, the proof is also valid for $\overline{\Delta} < \beta \cdot \Delta$, and for $\overline{\Delta} > \beta \cdot \Delta$ in which $Y^{tot} \geq \frac{\overline{\Delta} - \Delta}{\beta - 1}$. In the case of β<1, the proof is also valid for $\overline{\Delta} > \beta \cdot \Delta$, and for $\overline{\Delta} < \beta \cdot \Delta$ in which $Y^{tot} \leq \frac{\overline{\Delta} - \Delta}{\beta - 1}$. Note that if $\Delta << Y^{tot}$, none of the assumptions above is needed. Using $\overline{\Delta} \cong \beta \cdot \Delta$ we get that: $A \cong 1 \quad ; \quad B \cong \beta - 1$.

In the case of over expression (β > 1), B > 0, and therefore, 5.16 can be reduced to $\overline{X} \geq \beta \cdot X$. Substituting M back to the equations we get:

5.17. $$\overline{M} \leq \frac{1}{\beta} \cdot M$$

In the case of inhibition (β < 1), B < 0, and therefore, 5.16 can be reduced to $\overline{X} \leq \beta \cdot X$. Substituting M back to the equations we get:

5.18. $$\overline{M} \geq \frac{1}{\beta} \cdot M$$

**Q.E.D.**

If a positive feedback branch was the mechanism which generates a Yan degradation border, the critical MAPK concentration which defines the border will decrease upon Yan over expression. Since the MAPK concentration is a monotonically decreasing function of the distance from the midline, Yan degradation border location will move away from the midline as Yan is over-expressed. We can rule out the possibility that a positive feedback branch is responsible for the Yan degradation border, as its sensitivity to over expression contradicts our experimental observations.

As we discussed in the main text, at the cooperative first order case the location of Yan degradation border is significantly less sensitive to over expression than the non-cooperative case. This can be seen using the equations above simply by transforming M → $M^n$, where n designate the hill coefficient. Using equations 5.17 and 5.18 will result in:

5.19. $$\overline{M} \leq \left(\frac{1}{\beta}\right)^{\frac{1}{n}} \cdot M \qquad , for\ \beta > 1$$

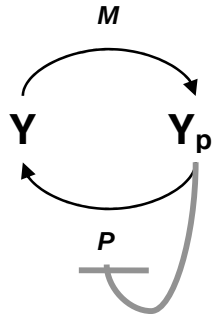
$$\overline{M} \geq \left(\frac{1}{\beta}\right)^{\frac{1}{n}} \cdot M \qquad , for\ \beta < 1$$

From 5.19 it is clear that this constraint is much more significant for small n, as seen in the simulations in the main text (Fig. 4).

## 5.c. An example of a network with positive feedback

In this section we will introduce an example for a first-order positive feedback network. Section 5.c.i. will discuss the steady-state solution of the network, and check the general proof in section 5.b. for this system. At the end of the section, we will find the parameter values that generate a switch-like behavior, and discuss the sensitivity of the critical MAPK levels to over expression. Section 5.c.ii. will discuss the stability of the steady-state solutions, and the possible oscillations are discussed in section 5.c.iii. The dynamics of the system will be discussed in chapter 6.

Consider the following network:




M and P designate the active MAPK and phosphatase concentrations, respectively. As the output ($Y_p$) is increased, the dephosphorylation rate decreases, and therefore, the output concentration is increased – The feedback branch is positive. The following equation describes the quasi-steady-state of such a network:

5.20. $$\frac{dY_p}{dt} = k_m \cdot \frac{[MAPK^*]}{1+\frac{K_m}{Y}} - k_p \cdot \frac{K_f^n}{K_f^n + Y_p^n} \cdot \frac{[P]}{1+\frac{K_p}{Y_p}}$$

, where a positive feedback term: $\frac{K_f^n}{K_f^n + Y_p^n}$ was added. There are two interesting limits of this term:

***For $K_f^n >> Y_p^n$:***

$\frac{K_f^n}{K_f^n + Y_p^n} \to 1$, and we are back to the regular first order kinetics without a feedback branch.

***For $Y_p^n >> K_f^n$:***

$\frac{K_f^n}{K_f^n + Y_p^n} \to \left(\frac{K_f}{Y_p}\right)^n$. In this case the positive feedback can dominate the kinetics. It will be more dominant as n increases. This limit can be reached only if $\left(Y^{tot}\right)^n >> K_f^n$.

Note that the range of $Y_p$ which is between these limits ($Y_p^n = O\left(K_f^n\right)$) gets smaller as n is increased, which makes the transition between these two states faster.
At first order, equation 5.20 reduces to:

5.21. $$\frac{dY_p}{dt} = C_m \cdot [MAPK^*] \cdot Y - C_p \cdot Y_p \quad ; \quad C_m \equiv \frac{k_m}{K_m} \quad ; \quad C_p \equiv \frac{k_p}{K_p}[P] \cdot \frac{K_f^n}{K_f^n + Y_p^n}$$

## 5.c.i. Steady-state solution

For simplicity, we will neglect the concentration of the complexes with respect to the free substrate, such that: $Y^{tot} = Y + Y_p + \Delta \cong Y + Y_p$. The effect of the complexes ($\Delta$) can be considered simply by transforming $Y^{tot} \rightarrow (Y^{tot} - \Delta)$ in the following derivation.
Using the above conservation equation, the general steady-state solution of 5.21 is:

5.22. $$Y_p \cdot \left( [MAPK^*] + \nu \cdot \frac{K_f^n}{K_f^n + Y_p^n} \right) = [MAPK^*] \cdot Y^{tot} \quad ; \quad \nu \equiv \frac{K_m}{k_m} \cdot \frac{k_p}{K_p} \cdot [P]$$

According to the claim in section 5.b., this network should be sensitive to over expression, such that upon over expression $Y^{tot} \rightarrow \beta \cdot Y^{tot}$, $Y_p$ will stay unchanged only if the input ($[MAPK^*]$) is changed at least by a factor of $\beta$. In order to check this claim we will rewrite equation 5.22 as:

5.23. $$[MAPK^*] = \nu \cdot Y_p \frac{K_f^n}{K_f^n + Y_p^n} \cdot \frac{1}{Y^{tot} - Y_p}$$

Upon over expression: $Y^{tot} \rightarrow \beta \cdot Y^{tot}$, $Y_p \rightarrow Y_p$, the new MAPK value ($\overline{M}$) will be:

5.24. $$\overline{M} = \nu \cdot Y_p \frac{K_f^n}{K_f^n + Y_p^n} \cdot \frac{1}{\beta \cdot Y^{tot} - Y_p}$$

Using 5.23 and 5.24 in the over expression case ($\beta > 1$), it turns out that $\overline{M} < \frac{[MAPK^*]}{\beta}$, i.e. the input is decreased at least by a factor of $\beta$. In case of inhibition ($\beta < 1$), it turns out that: $\overline{M} > \frac{[MAPK^*]}{\beta}$, i.e. the input is increased at least by a factor of $1/\beta$.

In order to understand the network steady-state behavior, let us check the steady-state solution in the extreme limits of equation 5.22:

**For $K_f^n >> Y_p^n$:**

5.25. $$Y_p = \frac{[MAPK^*]}{[MAPK^*] + \nu} \cdot Y^{tot}$$

$$\rightarrow \qquad Y_p \xrightarrow{[MAPK^*] << \nu} \frac{[MAPK^*]}{\nu} \cdot Y^{tot} << Y^{tot} \quad ; \quad Y_p \xrightarrow{[MAPK^*] >> \nu} Y^{tot}$$

**For $Y_p^n >> K_f^n$:**

5.26. $$Y_p = Y^{tot} - K_f \cdot \left(\frac{K_f}{Y_p}\right)^{n-1} \cdot \frac{\nu}{[MAPK^*]}$$

$$\rightarrow \quad Y_p \xrightarrow{[MAPK^*] \to \nu \cdot \left(\frac{K_f}{Y^{tot}}\right)\left(\frac{K_f}{Y_p}\right)^{n-1}} 0 \;\; ; \;\; Y_p \xrightarrow{[MAPK^*] >> \nu \cdot \left(\frac{K_f}{Y^{tot}}\right)\left(\frac{K_f}{Y_p}\right)^{n-1}} Y^{tot}$$

The equations above show that at different active MAPK limits, the output ($Y_p$) changes dramatically. The important thresholds are the following:

5.27. $$A) \;\; Y_p^n = K_f^n$$
$$B) \;\; [MAPK^*] = \nu$$
$$C) \;\; [MAPK^*] = \nu \cdot \left(\frac{K_f}{Y^{tot}}\right) \cdot \left(\frac{K_f}{Y_p}\right)^{n-1}$$

,where $Y_p$ increases monotonically with the MAPK level. This network can generate a switch if the system can jump between two output states: $Y_p \rightarrow 0$ and $Y_p \rightarrow Y^{tot}$ (large $Y_p$ range), with a small input transition (small $[MAPK^*]$ range). It is important to mention that for large n values, thresholds 5.27.A. and 5.27.C. can be passed with a small change in MAPK value.

The sensitivity of the threshold to over expression can be inferred by checking its dependence on $Y^{tot}$. For example, the threshold 5.27.C. depends on $\left(Y^{tot}\right)^{-1}$, and therefore, it is sensitive to over-expression.

As we mentioned before, The feedback loop is relevant only if $\left(Y^{tot}\right)^n >> K_f^n$, since this is required for reaching the limit: $Y_p^n >> K_f^n$. In the following we will discuss the steady-state solution of this case, for different parameter ranges, using equations 5.25 and 5.26.

**Define:** $T_f$ = The active MAPK level for which $Y_p = K_f$.
If $[MAPK^*] >> T_f$ then $Y_p >> K_f$, and vice versa.

**(A) $T_f >>> \nu$ , and there is a range where $T_f >> [MAPK^*] >> \nu$**

5.28. $$Y_p = \begin{cases} \frac{[MAPK^*]}{\nu} \cdot Y^{tot} << Y^{tot} & , \quad 0 \le [MAPK^*] << \nu \\ Y^{tot} & , \quad \nu << [MAPK^*] << T_f \\ Y^{tot} & , \quad [MAPK^*] \ge O(T_f) \end{cases}$$

**(B)** $T_f \geq O(\nu)$ **, but there is no range were** $T_f >> [MAPK^*] >> \nu$

$$5.29. \qquad Y_p = \begin{cases} \dfrac{[MAPK^*]}{\nu} \cdot Y^{tot} << Y^{tot} & , \quad 0 \leq [MAPK^*] << \nu \\ O\left(Y^{tot}\right) & , \quad O(\nu) \leq [MAPK^*] \leq O\left(T_f\right) \\ Y^{tot} & , \quad [MAPK^*] >> T_f \end{cases}$$

**(C)** $T_f << \nu$ **, but** $\nu$ **is small enough such that for all** $[MAPK^*] >> T_f$**, we get:**

$$[MAPK^*] >> \nu \cdot \left(\frac{K_f}{Y^{tot}}\right) \cdot \left(\frac{K_f}{Y_p}\right)^{n-1}$$

$$5.30. \qquad Y_p = \begin{cases} \dfrac{[MAPK^*]}{\nu} \cdot Y^{tot} << Y^{tot} & , \quad 0 \leq [MAPK^*] << T_f \\ Y^{tot} & , \quad [MAPK^*] >> T_f \end{cases}$$

**(D)** $T_f <<< \nu$ **, and** $\nu$ **is large enough for the following limit to exist:**

$$T_f << [MAPK^*] = O\left(\nu \cdot \left(\frac{K_f}{Y^{tot}}\right) \cdot \left(\frac{K_f}{Y_p}\right)^{n-1}\right) \quad , but \quad [MAPK^*] > \nu \cdot \left(\frac{K_f}{Y^{tot}}\right) \cdot \left(\frac{K_f}{Y_p}\right)^{n-1}$$

$$5.31. \qquad Y_p = \begin{cases} \dfrac{[MAPK^*]}{\nu} \cdot Y^{tot} << Y^{tot} & , \quad 0 \leq [MAPK^*] << T_f \\ Y^{tot} - \dfrac{\nu \cdot K_f}{[MAPK^*]} \cdot \left(\dfrac{K_f}{Y_p}\right)^{n-1} << Y^{tot} & , \quad T_f << [MAPK^*] = O\left(\nu \cdot \left(\dfrac{K_f}{Y^{tot}}\right) \cdot \left(\dfrac{K_f}{Y_p}\right)^{n-1}\right) \\ Y^{tot} & , \quad [MAPK^*] >> \nu \cdot \left(\dfrac{K_f}{Y^{tot}}\right) \cdot \left(\dfrac{K_f}{Y_p}\right)^{n-1} \end{cases}$$

**(E)** $T_f$ **<<<** $\nu$ **, and** $\nu$ **is very large such that the following limit to exist:**

$$T_f << [MAPK^*] < \nu \cdot \left(\frac{K_f}{Y^{tot}}\right) \cdot \left(\frac{K_f}{Y_p}\right)^{n-1}$$

5.32.
$$Y_p = \begin{cases} \dfrac{[MAPK^*]}{\nu} \cdot Y^{tot} << Y^{tot} & , \quad 0 \le [MAPK^*] << T_f \\ oscillations & , \quad T_f << [MAPK^*] < \nu \cdot \left(\dfrac{K_f}{Y^{tot}}\right) \cdot \left(\dfrac{K_f}{Y_p}\right)^{n-1} \\ << Y^{tot} & , \quad \nu \cdot \left(\dfrac{K_f}{Y^{tot}}\right) \cdot \left(\dfrac{K_f}{Y_p}\right)^{n-1} < [MAPK^*] = O\left(\nu \cdot \left(\dfrac{K_f}{Y^{tot}}\right) \cdot \left(\dfrac{K_f}{Y_p}\right)^{n-1}\right) \\ Y^{tot} & , \quad [MAPK^*] >> \nu \cdot \left(\dfrac{K_f}{Y^{tot}}\right) \cdot \left(\dfrac{K_f}{Y_p}\right)^{n-1} \end{cases}$$

As we mentioned before, A switch is generated if the output jumps between two opposite states (large output range) with a small input transition (small input range). Since the threshold in (A) is $[MAPK^*] = \nu$, the change in input needed for transitioning between the opposing states is too large to generate a switch. In (B) the threshold occurs at the MAPK value for which $Y_p^n = K_f^n$, and therefore, the input range is small for large n. However, the differences between these output states is small (from $O(Y^{tot})$ to $Y^{tot}$), and therefore, a very moderate switch will be generated.

In (C) a switch is generated if n is large, since the threshold occurs at the MAPK value for which $Y_p^n = K_f^n$, and the output range is large. In (D) a switch is also generated for large n, since the threshold: $[MAPK^*] = \nu \cdot \left(\frac{K_f}{Y^{tot}}\right) \cdot \left(\frac{K_f}{Y_p}\right)^{n-1}$ can be passed with a small increase in MAPK level, and the output range is large. In the special case of (E) no stable steady-state is possible in the middle range, due to oscillations which will be discussed later on. Thus, only cases (C) and (D) can generate a switch.

We can summarize the parameter range needed for generating a switch in the following: $Y^{tot} >> K_f$, large n, and $T_f << \nu$ ($K_f << \nu$). Using these limits in numerical simulations of Yan degradation network with positive feedback also exhibits a switch-like response, and agrees with these analytical expectations (Figs. 3, S3C).

In the derivation above the complex concentrations were neglected with respect to the free substrate such that: $Y^{tot} \cong Y + Y_p$. We can study the effect of the complexes ($\Delta$) by transforming $Y^{tot} \rightarrow (Y^{tot} - \Delta)$ in the equations above. As the complex concentrations increase with respect to the substrate, the differences between the output states decrease, and therefore, the switch will become more moderate.

In (C) the critical active MAPK level ($M_C$) is the level for which: $Y_p = K_f$. According to 5.30, at low MAPK values: $Y_p = \frac{[MAPK^*]}{\nu} \cdot Y^{tot}$. When we reach the threshold ([MAPK$^*$]=$M_c$) we get: $Y_p = K_f = \frac{M_C}{\nu} \cdot Y^{tot}$, or put another way: $M_C = \nu \cdot \frac{K_f}{Y^{tot}} \propto \left(Y^{tot}\right)^{-1}$. In (D) the critical active MAPK level is: $M_C \equiv \nu \cdot \left(\frac{K_f}{Y^{tot}}\right) \cdot \left(\frac{K_f}{Y_p}\right)^{n-1} \propto \left(Y^{tot}\right)^{-1}$. Since these two thresholds depend on $Y^{tot}$, they are sensitive to Yan over expression.

Fig. 4C shows the steady-state Yan profile of a positive feedback network upon over expression. Indeed, a clear sensitivity of Yan degradation border to over expression was observed. Moreover, in Fig 4E the threshold MAPK level was proportional to $\left(Y^{tot}\right)^{-1.016}$ in more than 99.56% confidence (The adjusted R-square value in Matlab fit function), which is in complete correspondence to our analytical findings.

The steady-state solutions of the four models are summarized in figure S3 and table 1:

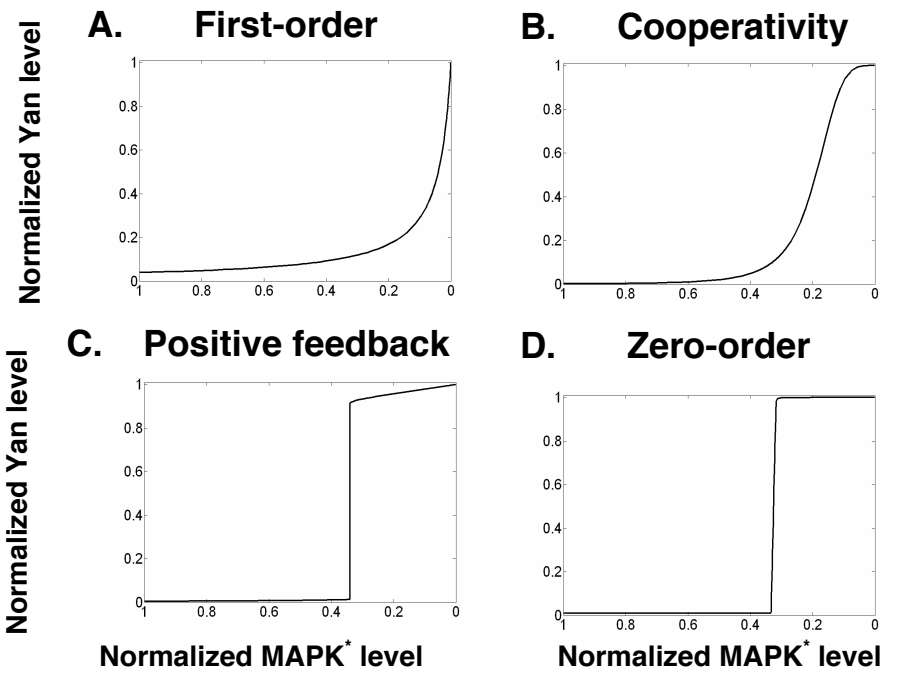



**Figure S3.** Zero order networks and first order networks with cooperativity or positive feedback, can generate a sharp threshold of Yan degradation.

Yan steady state concentration was derived by numerical simulations (see Methods for parameters used). The concentration levels are normalized with the corresponding maximal concentrations. At first-order, there is a smooth gradual increase in the total Yan level as a function of a decrease in the total activated MAPK level. However, at cooperative first-order with n = 4 a sharp transition in Yan level is observed. Zero-order kinetics and systems with positive feedback exhibit a sharp threshold as well.

| Model | First order | positive feedback | Zero order |
|---|---|---|---|
| **Steady-state solution** | $Y=\frac{\alpha/\lambda}{1+\frac{[MAPK^*]^n}{C_n}}$<br>$Y_p=\frac{\alpha/\lambda_p}{1+\frac{C_n}{[MAPK^*]^n}}$<br>$C_n$= constant | $Y_p=\begin{cases}0 & ,[MAPK^*]^l << T_f{}^l \\ Y^{tot} & ,[MAPK^*]^l >> T_f{}^l\end{cases}$<br>$T_f$ is the active MAPK level for which $Y_p = K_f$ (*). | $Y^{tot}=\begin{cases}\frac{\alpha}{\lambda} & ,[MAPK^*]<T_1 \\ \frac{\alpha}{\lambda_p} & ,[MAPK^*]>T_2\end{cases}$<br>$T_1\equiv\frac{k_p[P]}{k_m}$ ; $T_2\equiv\frac{\alpha+k_p[P]}{k_m}$ |
| **Conditions generating switch** | Large n | Large l, $K_f << Y^{tot}$, and $T_f << \nu$, where $\nu$ =const. | $\alpha << k_p[P]$ |
| **Sensitivity to over expression (factor β)** | MAPK is changed at least by a factor of $\beta^{\frac{1}{n}}$.<br>→ Sensitive only for small n. | MAPK is changed at least by a factor of β. | Not sensitive |

**Table 1**. Analytical solutions of Yan steady state distribution.

(*) The system was analyzed for a simple covalent modification system. The solution is stable for sufficiently small $K_f$.

## 5.c.ii. Stability of the steady-state

The stability of the steady-state solutions obtained in the previous section can be determined by linearizing about the fixed points. Using the quasi steady-state conservation equation:

5.33. $$Y^{tot}=Y+Y_p+[YMAPK^*]+[Y_PP]=Y\cdot\left(1+\frac{[MAPK^*]}{K_m}\right)+Y_p\cdot\left(1+\frac{[P]}{K_p}\right)$$

, the network equation 5.21 can be rewritten as:

5.34. $$\frac{dY_p}{dt}=A-B\cdot Y_p-C\cdot\frac{Y_p}{K_f^n+Y_p^n}\equiv f(Y_p)$$

$$A\equiv\frac{k_m\cdot[MAPK^*]}{1+\frac{K_m}{[MAPK^*]}}\cdot Y^{tot} \quad ; \quad B\equiv\frac{k_m\cdot[MAPK^*]}{1+\frac{K_m}{[MAPK^*]}}\cdot\left(1+\frac{[P]}{K_p}\right) \quad ; \quad C\equiv\frac{k_p}{K_p}[P]\cdot K_f^n$$

Consider a small perturbation (η) from the fixed point $\bar{Y}_p$: $Y_p=\bar{Y}_p+\eta$. Using Tailor expansion, and since $f(\bar{Y}_p)=0$, we get that $f(Y_p)\cong\eta\cdot f'(\bar{Y}_p)$, for cases where $f'(\bar{Y}_p)\neq 0$. Substituting in 5.34 will result in:

5.35. $$\frac{d\eta}{dt}=\eta\cdot f'(\bar{Y}_p)\quad\rightarrow\quad\eta=const\cdot e^{f'(\bar{Y}_p)\cdot t}$$

Therefore, the stability of the steady-state $\overline{Y}_p$ is determined by $f'(\overline{Y}_p)$: if $f'(\overline{Y}_p)<0$ then $\overline{Y}_p$ is a stable steady-state, whereas if $f'(\overline{Y}_p)>0$ then $\overline{Y}_p$ is non-stable. Since

5.36. $$f'(\overline{Y}_p)=C\cdot\frac{(n-1)\cdot\overline{Y}_p^{\,n}-K_f^n}{\left(K_f^n+\overline{Y}_p^{\,n}\right)^2}-B$$

, the active MAPK value for which $f'(\overline{Y}_p)=0$ is:

5.37. $$[MAPK^*]\Big|_{f'(\overline{Y}_p)=0}=\frac{(n-1)\cdot\overline{Y}_p^{\,n}-K_f^n}{\left(K_f^n+\overline{Y}_p^{\,n}\right)^2}\cdot K_f^n\cdot\nu\cdot\theta$$

$$\nu\equiv\frac{K_m}{k_m}\cdot\frac{k_p}{K_p}[P]\qquad;\qquad\theta\equiv\frac{1+\dfrac{[MAPK^*]\Big|_{f'(\overline{Y}_p)=0}}{K_m}}{1+\dfrac{[P]}{K_p}}$$

Note that θ represent the complexes contribution, and $\theta\to 1$ when the complex concentrations are negligible. Let us study the system at the two limits:

5.38. $$[MAPK^*]\Big|_{f'(\overline{Y}_p)=0}\xrightarrow{\overline{Y}_p^{\,n}<<K_f^n}-\nu\cdot\theta\quad<0$$

$$[MAPK^*]\Big|_{f'(\overline{Y}_p)=0}\xrightarrow{\overline{Y}_p^{\,n}>>K_f^n}(n-1)\cdot\left(\frac{K_f}{\overline{Y}_p}\right)^n\cdot\nu\cdot\theta\quad>0$$

When $\overline{Y}_p^{\,n}<<K_f^n$, the MAPK value is always larger than $[MAPK^*]\Big|_{f'(\overline{Y}_p)=0}$, and therefore, $f'(\overline{Y}_p)<0$. We can therefore conclude that the regular first-order solution without positive feedback is always stable. When $\overline{Y}_p^{\,n}>>K_f^n$, the problem is refined to two optional behaviors:

A. If the MAPK concentration is large enough such that both $\overline{Y}_p^{\,n}>>K_f^n$, and $[MAPK^*]>[MAPK^*]\Big|_{f'(\overline{Y}_p)=0}$, then $f'(\overline{Y}_p)<0$, and the fixed point $\overline{Y}_p$ is stable.

B. If some intermediate MAPK range exists where $\overline{Y}_p^{\,n}>>K_f^n$ but $[MAPK^*]<[MAPK^*]\Big|_{f'(\overline{Y}_p)=0}$, then $f'(\overline{Y}_p)>0$, and the fixed point $\overline{Y}_p$ is not stable. In this case we will get an oscillating solution, as discussed in section 5.c.iii.

When $\overline{Y}_p\cong K_f$ the threshold MAPK value is: $[MAPK^*]\Big|_{f'(\overline{Y}_p)=0}\xrightarrow{\overline{Y}_p\cong K_f}\frac{n-2}{4}\cdot\nu\cdot\theta$ .

For n = 1 the network is always stable as in the $\overline{Y}_p^{\,n}<<K_f^n$ case. However, for n > 2 the network

resembles the $\bar{Y}_p^n >> K_f^n$ case in which the threshold MAPK is positive, and therefore, the stability of the fixed points depends on the network input MAPK value. Note that we are interested in cases where a sharp switch is generated, and therefore, our network behaves like an n > 2 network.

Note that the MAPK limits mentioned above correspond to the limits of the steady-state solutions introduced in section 5.c.ii. (in 5.c.ii. the complexes contribution was neglected). It is also important to mention that the threshold $f'(\bar{Y}_p) = 0$ is a function of the input MAPK, and of the total Yan concentration ($\bar{Y}_p$ is a function of $Y^{tot}$). Thus, a change in [$MAPK^*$] and/or in $Y^{tot}$ can affect the stability of the steady-state.

This analytical solution can be represented graphically in phase plane, as shown in figure S4.

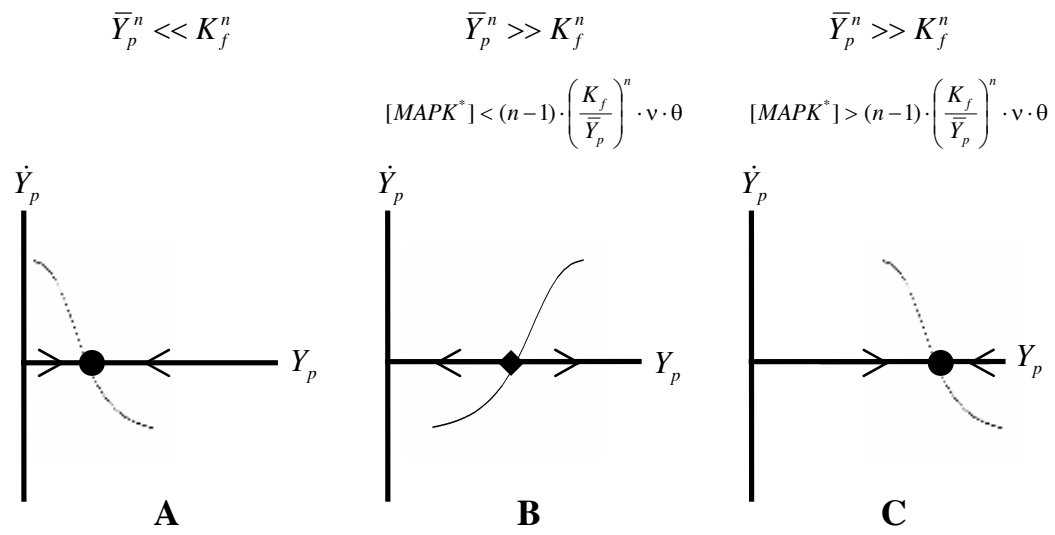



**Figure S4.** A first-order network with positive feedback has a non-stable steady state at a specific range of parameters.

When $\bar{Y}_p^n << K_f^n$, the fixed point is stable as expected for a first-order network without positive feedback (A). When the positive feedback is dominant ($\bar{Y}_p^n >> K_f^n$), the system can reach a non-stable steady state (B) or a stable one (C). The system can flip between cases B and C as the threshold parameters are changed. In particular, the system behavior is sensitive to over expression ($Y^{tot}$) and to the input values ([$MAPK^*$]).

## 5.c.iii. Oscillations

In section 5.c.ii. we encountered a region of MAPK for which the solution is not stable. In order to understand the kinetics in this region, let's consider an example network for which initially all Yan is non-phosphorylated, n>2, and the network input is:

$[MAPK^*] = \frac{1}{2}\nu \cdot \frac{K_f}{Y^{tot}} - \delta M$ , where $\delta M << \nu \cdot \frac{K_f}{Y^{tot}}$ is a small MAPK$^*$ value.

The network's initial condition is $Y_p^n << K_f^n$, and therefore, its dynamics is determined by:

5.39. $$\frac{dY_p}{dt} = \frac{k_m}{K_m}[MAPK^*] \cdot Y^{tot} - \frac{k_p}{K_p}[P] \cdot Y_p \quad ; \quad \frac{dY}{dt} = -\frac{dY_p}{dt}$$

, where $Y \cong Y^{tot} - Y_p \to Y^{tot}$ was used. In this limit, $Y_p$ will increase if $[MAPK^*] > \nu \cdot \frac{Y_p}{Y^{tot}} \equiv M_C^{(1)}$. Since $Y_p < K_f$, it is clear that $[MAPK^*] > M_C^{(1)}$. Therefore, $Y_p$ *increases* at least up to $O(K_f)$, where the positive feedback branch starts to affect the dynamics.

When $Y_p \cong K_f$ ($Y \cong Y^{tot}$) the dynamics is determined by:

5.40. $$\frac{dY_p}{dt} \cong \frac{k_m}{K_m}[MAPK^*] \cdot Y^{tot} - \frac{1}{2} \cdot \frac{k_p}{K_p}[P] \cdot Y_p \quad ; \quad \frac{dY}{dt} = -\frac{dY_p}{dt}$$

In this case, $Y_p$ will increase only if $[MAPK^*] > \frac{1}{2}\nu \cdot \frac{Y_p}{Y^{tot}} = \frac{1}{2}\nu \cdot \frac{K_f}{Y^{tot}} \equiv M_C^{(2)}$. Since in our case $[MAPK^*] < M_C^{(2)}$, *$Y_p$ will decrease*.

We might think that a steady-state for which: $[MAPK^*] = \frac{1}{2}\nu \cdot \frac{Y_p}{Y^{tot}} = \frac{1}{2}\nu \cdot \frac{K_f}{Y^{tot}} - \delta M$ , can be reached for $Y_p$ values which are slightly smaller than $K_f$. However, since $K_f << Y^{tot}$ and $n > 2$, this steady-state will fulfill:

5.41. $$\overline{Y}_p \cong K_f \quad ; \quad [MAPK^*] < \frac{n-2}{4} \cdot \nu \cdot \theta$$

, and therefore, the steady-state will not be stable (see 5.c.ii.).

The network output is bound between a lower limit in which the output increases and an upper limit in which it decreases, with no stable steady-state in between. Therefore, oscillations occur and no stable steady-state is reached.

A positive feedback network for which the steady-state $\overline{Y}_p$ satisfies: $\overline{Y}_p^n >> K_f^n$ and $[MAPK^*] < (n-1) \cdot \left(\frac{K_f}{\overline{Y}_p}\right)^n \cdot \nu \cdot \theta \equiv M_C$, reaches an unstable steady-state (Fig. S4.B), and

therefore, it oscillates. Note that since $Y_p$ increases monotonically with $Y^{tot}$, $M_C$ increases as $Y^{tot}$ is decreased.

Consider a network for which $\bar{Y}_p^n >> K_f^n$, but $[MAPK^*] > M_C$. This network will have a stable steady-state and will not oscillate (Fig. S4.C). If $Y^{tot}$ and/or MAPK is decreased the network can reach $[MAPK^*] < M_C$, and therefore, starts to oscillate.

This analytical prediction can be tested numerically in phase plane. The network defined by equation 5.21. was simulated using Matlab ODE15s function (Fig. S5). For the wild type network we used parameters which generate a switch (Fig. S5A). Then, we examined the network behavior in phase plane upon a decrease in $Y^{tot}$ (Fig. S5B), and a decrease in MAPK level (Fig. S5C). As expected, the wild type network (Figs. S5B, C- blue curves) has one single stable steady-state at a large $Y_p$ value. As the levels of MAPK or $Y^{tot}$ decreased, a non-stable steady-state was generated, and for very low levels (Figs. S5B, C- red curves) there was a single stable steady-state for which $Y_p << Y^{tot}$. These observations agree with our analytical findings.

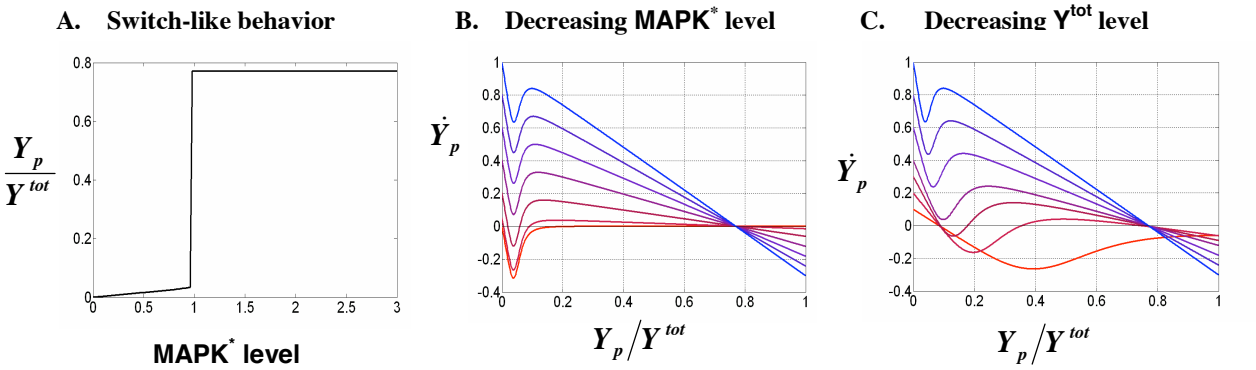



**Figure S5.** Steady states of a first-order network with positive feedback and stability analysis in phase plane.

Equation 5.21. was simulated using MATLAB ODE15s function, assuming the wild type network has the following first-order parameters: $k_m$ = 1 $s^{-1}$, $K_m$ = 100 $\mu$M, $k_p$ = 1 $s^{-1}$, $K_p$ = 100 $\mu$M, $K_f$ = 0.05 $\mu$M, n = 5, [P] = 30 $\mu$M, $Y^{tot}$ = 1 $\mu$M, [$MAPK^*$] = 3 $\mu$M. Figure A. shows the switch like behavior of the wild type network. Figure B. and C. examine the phase plane upon a decrease in MAPK value (Fig B; [$MAPK^*$] is multiplied by: $10^{-5}$, 0.05, 0.2, 0.4, 0.6, 0.8 and 1) and a decrease in the total Yan value (Fig C; $Y^{tot}$ is multiplied by: 0.1, 0.2, 0.3, 0.4, 0.6, 0.8 and 1). The wild type network (blue curve) has a single stable steady state at a high $Y_p$ value. As the levels decrease (curves become red), a non- stable steady state is generated (the points were curves intersect with $\dot{Y}_p = 0$, with a positive slop). At the lowest level (pure red) the network has a single steady state at $Y_p << Y^{tot}$.

In the main text of the paper, a simulation of the residual Yan concentration versus Yan over expression value was presented (Fig. 5E). It was mentioned for the case of a positive feedback network that the simulation is valid only for over expression values larger than 0.6 fold. Indeed, at these large values a stable steady-state was reached as Yan was fully degraded (Fig. S6A). The reason for the abnormal behavior at lower $Y^{tot}$ values (over expression of 0.28 – 0.6 fold) stems from the oscillatory behavior of the network in this region (Fig. S5B). When $Y^{tot}$ values were very small (over expression values less than 0.28), the oscillations stopped, and no switch was generated (Fig S6C), since the positive feedback branch was not effective ($Y_p^n >> K_f^n$

was not reached, or reached only when $Y_p \sim Y^{tot}$). The numerical simulations clearly match our analytical predictions.

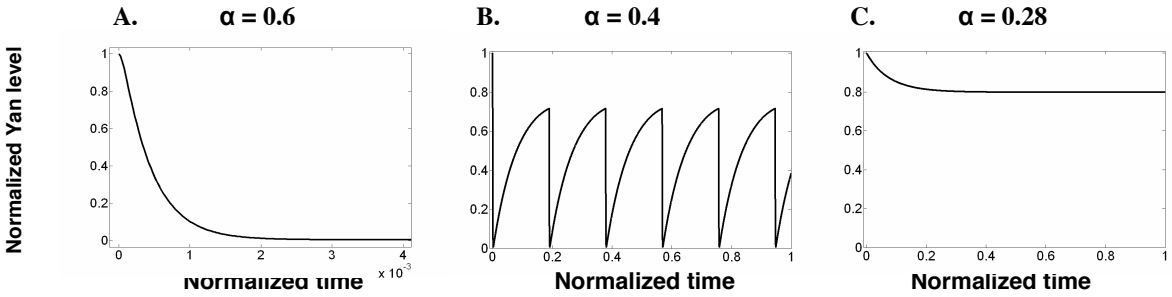



**Figure S6.** Dynamics of a first-order network with positive feedback at different levels of Yan expression.

This figure corresponds to figure 4E in the main text. The dynamics of the total Yan level is shown for different Yan production rates (α). The production rate values are normalized with respect to the wild type, and both axes are normalized to the maximal values. When the production rate is high ($\alpha > 0.6$), Yan is fully degraded and a stable steady state is reached (A). For small production rates ($0.28 < \alpha < 0.6$), Yan degrades and then start to oscillate (B). When Yan levels are very low ($\alpha < 0.28$), the positive feedback branch does not affect the kinetics, and Yan reach a stable steady state in which only a small part of Yan is degraded (C). For information about the parameters used see Methods.

# 6. Estimates for Yan Decay Time

To illustrate the dependence of the relaxation time on the level of Yan, we solved the dynamics for the simple case of the classical zero-order model at quasi steady-state (Equations 5.5 – 5.6).

## 6.a. First-order dynamics

At first-order kinetics ($Y^{tot} << K_m, K_p$) equations 5.5 and 5.6 reduce to:

6.1. $$\frac{dY_p}{dt} = \frac{k_m}{K_m}[MAPK^*]\cdot Y - \frac{k_p}{K_p}[P]\cdot Y_p \quad ; \quad \frac{dY}{dt} = -\frac{dY_p}{dt}$$

Using the quasi steady-state conservation equation:

6.2. $$Y^{tot} = Y + Y_p + [YMAPK^*] + [Y_P P] = Y\cdot\left(1+\frac{[MAPK^*]}{K_m}\right) + Y_p\cdot\left(1+\frac{[P]}{K_p}\right)$$

, the network will reach the following exponential time dependence:

6.3. $$Y_p(t) = C_1\cdot e^{-\frac{t}{T}} + C_2\cdot Y^{tot}$$ , where:

6.4. $$T \equiv \left(\frac{k_m\cdot[MAPK^*]}{K_m}\cdot\frac{1+\frac{[MAPK^*]}{K_m}}{1+\frac{[P]}{K_p}} + \frac{k_p\cdot[P]}{K_p}\right)^{-1}$$

$$C_2 \equiv \left(1+\frac{[P]}{K_p}+\frac{k_p\cdot[P]}{K_p}\cdot\frac{K_m}{k_m\cdot[MAPK^*]}\cdot\left(1+\frac{[MAPK^*]}{K_m}\right)\right)^{-1}$$

, and $C_1$ is a constant which depends on the network initial conditions. Note that the time scale (T) does not depend on the initial conditions, nor on $Y^{tot}$ levels. Thus, over expression of Yan will not change the network relaxation time. The cooperative first order case is similar to the non-cooperative one, since the transformation $([MAPK^*]/K_m)\rightarrow([MAPK^*]/K_m)^n$ can affect the constants (for example, the relaxation time), but not its exponential behavior.

Although Yan degradation network is more complicated than the classical model, the same qualitative behavior is observed (Figs. 5A,B).

## 6.b. Dynamics at first-order with positive feedback

As a simple example, we will estimate the dynamics of the network introduced in section 5.c. The quasi steady-state dynamics of this network is:

6.5. $$\frac{dY_p}{dt} = \frac{k_m}{K_m} \cdot [MAPK^*] \cdot Y - \frac{k_p}{K_p}[P] \cdot \frac{K_f^n}{K_f^n + Y_p^n} \cdot Y_p \quad ; \quad \frac{dY}{dt} = -\frac{dY_p}{dt}$$

When $Y_p << K_f$, equation 6.5 reduces to the regular first-order case without feedback (equation 6.1). Thus, at this limit the dynamics of the output ($Y_p$) is exponential with a time scale T (equation 6.4).

When $Y_p >> K_f$, equation 6.5 can be written as:

6.6. $$\frac{dY_p}{dt} = \frac{k_m}{K_m} \cdot [MAPK^*] \cdot Y - \frac{k_p}{K_p}[P] \cdot \frac{K_f^n}{Y_p^{n-1}} \quad ; \quad \frac{dY}{dt} = -\frac{dY_p}{dt}$$

Equation 6.6 consists of two terms. Whether the first one dominates the equation or the other, significantly affect the dynamics of the network. The first term will be dominant when the input value is:

6.7. $$[MAPK^*] >> \frac{K_m}{k_m}\frac{k_p}{K_p}[P] \cdot \frac{K_f}{Y^{tot}} \cdot \left(\frac{K_f}{Y_p}\right)^{n-1} \equiv \delta$$

Note that $\delta$ is a small number in this region ($Y_p >> K_f$), and for sufficiently small $K_f$ values equation 6.7 is fulfilled. The second term starts to affect the dynamics as [$MAPK^*$] reaches $\delta$. These limits correspond to the steady-state limits, which were discussed at chapter 5.c. $\left(\delta = \nu \cdot \frac{K_f}{Y^{tot}} \cdot \left(\frac{K_f}{Y_p}\right)^{n-1}\right)$.

First, let's assume that the first term in 6.6 dominates the equation dynamics. Using the conservation equation 6.2, it is clear that Y is proportional to $Y_p$, and therefore, $Y_p$ dynamics is exponential:

6.8. $$Y_p(t) = \tilde{C}_1 \cdot e^{-\frac{t}{\tilde{T}}} + \tilde{C}_2 \cdot Y^{tot}$$ , where

6.9. $$\widetilde{T} \equiv \left( \frac{k_m \cdot [MAPK^*]}{K_m} \cdot \frac{1+\frac{[MAPK^*]}{K_m}}{1+\frac{[P]}{K_p}} \right)^{-1}$$

$$\widetilde{C}_2 \equiv \left(1+\frac{[P]}{K_p}\right)^{-1}$$

, and $\widetilde{C}_1$ is determined by the initial conditions. Note that the time scale $\widetilde{T}$ is larger than the first-order one (*T)*. Therefore, the time to reach steady-state increases.

Consider a feedback network with the following input: $MAPK > \nu$ and $MAPK >> \delta$ when $Y_p >> K_f$, where $\nu = \frac{K_m}{k_m}\frac{k_p}{K_p}[P]$. If the network starts from an initial condition: Y(0) = $Y^{tot}$, $Y_p(0) = 0$, the network dynamics will be as follows: $Y_p$ will increase exponentially with a time scale T. This time scale will increase as $Y_p = O(K_f)$ is reached, to a maximal time scale of $\widetilde{T}$ at $Y_p >> K_f$. Therefore, $Y_p$ always increases exponentially, but the network shifts to a larger exponential time scale at the threshold $Y_p = K_f$.

Consider a network with the following initial conditions: Y(0) = $Y^{tot}$, $Y_p(0) = 0$. The system dynamics will start as a simple first order one:

6.10. $$Y_p(t) = C_2 \cdot Y^{tot}\left(1-e^{-\frac{t}{T}}\right)$$

, where $C_2$ is the constant given by equation (6.4).The time to reach the threshold $Y_p = K_f$ is:

6.11. $$t_f = T\ln\left(\left(1-\frac{K_f}{C_2 \cdot Y^{tot}}\right)^{-1}\right)$$

Therefore, upon Yan over expression (an increase in $Y^{tot}$) $t_f$ will decrease and the system will pass the threshold $Y_p = K_f$ faster. This way the system's exponential time scale will increase at an earlier stage and the relaxation time of the system will increase. Note, however, that since the dependence in $Y^{tot}$ is logarithmic, we can expect that the increase in relaxation time upon over-expression will be a moderate one. Our numerical simulations fit these analytical predictions (Fig. 5.C).

Let's consider the opposite limit where the second term in 6.6 is larger than the first term. This occurs when there is a MAPK value for which $MAPK < \delta$ when $Y_p >> K_f$. In section 5.c.ii. and 5.c.iii. we found that this MAPK level generates oscillations. In section 5.c.ii. and 5.c.iii. we showed that oscillations can also occur between the $Y_p >> K_f$ range and the $Y_p = O(K_f)$ range. These oscillations were detected in out numerical simulation at low $Y^{tot}$ values, as discussed in figure S6.

## 6.c. Zero-order dynamics

At zero-order kinetics ($Y^{tot} >> K_m, K_p$) when Y, $Y_p = O(Y^{tot})$, equations 5.5 and 5.6 can be rewritten as:

6.12. $$\frac{dY_p}{dt} = k_m[MAPK^*] - k_p[P] \quad ; \quad \frac{dY}{dt} = -\frac{dY_p}{dt}$$

, which result in the following linear time dependence:

6.13. $$Y(t) = Y(0) - \left(k_m[MAPK^*] - k_p[P]\right)\cdot t \quad ; \quad Y_p(t) = Y_p(0) + \left(k_m[MAPK^*] - k_p[P]\right)\cdot t$$

Assuming $Y(0) = Y^{tot}$, $Y_p(0) = 0$ will result in:

6.14. $$Y(t) = Y^{tot} - \left(k_m[MAPK^*] - k_p[P]\right)\cdot t \quad ; \quad Y_p(t) = \left(k_m[MAPK^*] - k_p[P]\right)\cdot t$$

Note that the decay time depends linearly on $Y^{tot}$ level. Thus, at the zero-order kinetics we expect a longer relaxation time upon Yan over expression. In the Yan degradation network similar linear time dependence is observed (Fig. 5D).

The dynamics of the four models is summarized in table 2:

| Model | First order | Positive feedback | Zero order |
|---|---|---|---|
| **Yan dynamics** | $Y_p(t) = C_1 \cdot e^{-\frac{t}{T}} + C_2 \cdot Y^{tot}$<br>$C_1$, $C_2$ and T are constants<br>T doesn't depend on $Y^{tot}$ | $Y_p(t) = \begin{cases} C_1 \cdot e^{-\frac{t}{T}} + C_2 \cdot Y^{tot}, & [MAPK^*]^l << T_f^{\,l} \\ \tilde{C}_1 \cdot e^{-\frac{t}{\tilde{T}}} + \tilde{C}_2 \cdot Y^{tot}, & [MAPK^*]^l >> T_f^{\,l} \end{cases}$<br>$C_1$, $C_2$, T, $\tilde{C}_1$, $\tilde{C}_2$, and $\tilde{T}$ are constants<br>$\tilde{T} > T$ (*) | $Y_p(t) = \left(k_m[MAPK^*] - k_p[P]\right)\cdot t$<br>$Y(t) = Y^{tot} - \left(k_m[MAPK^*] - k_p[P]\right)\cdot t$<br>(**) |
| **Sensitivity to over expression (β)** | Not sensitive | The relaxation time slightly increases | The relaxation time increases linear in β |

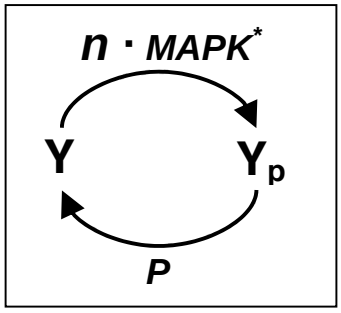
**Table 2**. Analytical solutions of Yan dynamics in a covalent modification system (Fig. 7A).

(*) $K_f$ is assumed to be sufficiently low. Note that for specific parameters range the positive feedback network can oscillate (chapter 5.c).

(**) Y, $Y_p = O(Y^{tot})$ was assumed. We used the biological problem initial conditions (The ventral ectoderm pattern at stage 9): $Y(0) = Y^{tot}$, $Y_p(0) = 0$.

# 7. Modeling Cooperative First Order Networks with Subsequent Bindings of Active MAPK to Yan

In the main text we formulated the cooperative first order case by assuming that n MAPK* molecules are binding simultaneously to Yan in order to form Yan-MAPK* complex. In this chapter we will discuss the alternative formulation where subsequent bindings of MAPK* to Yan are needed to generate the complex. For simplicity, we will discuss the serial binding case in the classical Goldbeter and Koshland covalent modification system, as shown below. In the following discussion we will show that our conclusion that cooperativity can not explain the experimental observations, is still valid when the alternative cooperativity formulation is used.




Modeling cooperativity as subsequent bindings of MAPK* molecules is based on the following reactions:

$$Y + M \underset{d_m^{(1)}}{\overset{a_m^{(1)}}{\rightleftharpoons}} YM^{(1)}$$

$$YM^{(1)} + M \underset{d_m^{(2)}}{\overset{a_m^{(2)}}{\rightleftharpoons}} YM^{(2)}$$

$$\vdots$$

$$YM^{(n-2)} + M \underset{d_m^{(n-1)}}{\overset{a_m^{(n-1)}}{\rightleftharpoons}} YM^{(n-1)}$$

} n-1 Equations

$$YM^{(n-1)} + M \underset{d_m^{(n)}}{\overset{a_m^{(n)}}{\rightleftharpoons}} YM^{(n)} \xrightarrow{k_m} Y_p + n\cdot M$$

$$Y_p + P \underset{d_p}{\overset{a_p}{\rightleftharpoons}} Y_pP \xrightarrow{k_p} Y + P$$

These reactions result in the following equations:

$$7.1.\quad \begin{cases} \frac{d[YM^{(1)}]}{dt} = a_m^1 \cdot Y \cdot M_f - d_m^1[YM^{(1)}] - a_m^2 \cdot [YM^{(1)}] \cdot M_f + d_m^2[YM^{(2)}] \\ \frac{d[YM^{(2)}]}{dt} = a_m^2 \cdot [YM^{(1)}] \cdot M_f - d_m^2[YM^{(2)}] - a_m^3 \cdot [YM^{(2)}] \cdot M_f + d_m^3[YM^{(3)}] \\ \vdots \\ \frac{d[YM^{(n-1)}]}{dt} = a_m^{n-1} \cdot [YM^{(n-2)}] \cdot M_f - d_m^{n-1}[YM^{(n-1)}] - a_m^n \cdot [YM^{(n-1)}] \cdot M_f + d_m^n[YM^{(n)}] \\ \frac{d[YM^{(n)}]}{dt} = a_m^n \cdot [YM^{(n-1)}] \cdot M_f - (d_m^n + k_m) \cdot [YM^{(n)}] \end{cases}$$

$$7.2\quad \frac{d[Y_P P]}{dt} = a_P \cdot Y_P \cdot P_f - \left(d_P + k_P\right) \cdot [Y_P P]$$

$$7.3.\quad \frac{dY}{dt} = -a_m^1 \cdot Y \cdot M_f + d_m^1 \cdot [YM^{(1)}] + k_P \cdot [Y_P P]$$

$$7.4\quad \frac{dY_P}{dt} = -a_P \cdot Y_P \cdot P_f + d_P \cdot [Y_P P] + k_m \cdot [YM^{(n)}]$$

, and two enzyme conservation equations:

$$7.5.\quad [MAPK^*] = M_f + \sum_{k=1}^{n} k \cdot [YM^{(k)}]$$

$$7.6.\quad [P] = P_f + [Y_p P]$$

Y and $Y_P$ designate Yan in its non-phosphorylated and phosphorylated isoforms, respectively. $M_f$ and $P_f$ are the free MAPK* and phosphatase, respectively. $[MAPK^*]$ and [P] are the total levels of enzymes in both free and complex forms. $[YM^{(k)}]$ is a complex of Yan with k MAPK molecules. At quasi-steady state equations 7.1 and 7.2 reduce to:

$$7.7.\quad \begin{cases} a_m^1 \cdot Y \cdot M_f + d_m^2[YM^{(2)}] = d_m^1[YM^{(1)}] + a_m^2 \cdot [YM^{(1)}] \cdot M_f \\ a_m^2 \cdot [YM^{(1)}] \cdot M_f + d_m^3[YM^{(3)}] = d_m^2[YM^{(2)}] + a_m^3 \cdot [YM^{(2)}] \cdot M_f \\ \vdots \\ a_m^{n-1} \cdot [YM^{(n-2)}] \cdot M_f + d_m^n[YM^{(n)}] = d_m^{n-1}[YM^{(n-1)}] + a_m^n \cdot [YM^{(n-1)}] \cdot M_f \\ a_m^n \cdot [YM^{(n-1)}] \cdot M_f = (d_m^n + k_m) \cdot [YM^{(n)}] \end{cases}$$

$$7.8.\quad [Y_P P] = [P] \cdot \frac{Y_p}{Y_p + K_p} \quad ; \quad P_f = [P] \cdot \frac{K_p}{Y_p + K_p} \quad ; \quad K_p \equiv \frac{k_p + d_P}{a_P}$$

Equation 7.7 can be rewritten as follows:

7.9. $$[YM^{(k-2)}] = A^k \cdot [YM^{(k-1)}] - B^k \cdot [YM^{(k)}] \quad ; \quad 2 \le k \le n$$
$$[YM^{(n-1)}] \cong C \cdot [YM^{(n)}]$$
$$C \equiv \frac{K_m}{M_f} = \frac{d_m^n + k_m}{a_m^n \cdot M_f} \quad ; \quad A^k \equiv \frac{d_m^{k-1}}{a_m^{k-1} \cdot M_f} + \frac{a_m^k}{a_m^{k-1}} \quad ; \quad B^k \equiv \frac{d_m^k}{a_m^{k-1} \cdot M_f}$$

, where $YM^{(0)} \equiv Y$. These n equations can be used to find the dependence of each complex in Y and $M_f$ ($[YM^{(k)}] = f(Y, M_f), \quad 1 \le k \le n$). An important point we can realize from equation 7.9 is that the concentration of each Yan-MAPK* complex is linearly dependent on Yan levels. Put another way,

7.10. $$[YM^{(k)}] = \alpha^k(M_f) \cdot Y \qquad ; \quad 1 \le k \le n$$

, where $\alpha^k$ can be calculated from equation 7.9 At first order the level of Yan is limiting and therefore most enzymes are free: $M_f >> \sum_{k=1}^{n} k \cdot [YM^{(k)}]$, or $M_f \cong [MAPK^*]$. Consequently, $\alpha^k$ are only a function of the rate constants and the total amount of MAPK*. At first order $Y^{tot} >> K_P$ and therefore equation 7.8 can be rewritten as:

7.11. $$[Y_P P] \cong \frac{[P]}{K_p} \cdot Y_p \quad ; \quad P_f \cong P^{tot}$$

By substituting 7.10 and 7.11 into 7.3 and 7.4 we will get:

7.12. $$\frac{dY}{dt} = \left(-a_m^1 \cdot [MAPK^*] + d_m^1 \cdot \alpha^1\right) \cdot Y + \left(\frac{k_P \cdot [P]}{K_p}\right) \cdot Y_P$$

7.13 $$\frac{dY_P}{dt} = \left(\frac{-k_P \cdot [P]}{K_p}\right) \cdot Y_P + \left(k_m \cdot \alpha^n\right) \cdot Y$$

In our case the total amount of Yan is constant, namely:

7.14. $$Y^{tot} = Y + Y_P + [Y_P P] + \sum_{k=1}^{n} [YM^{(k)}]$$

Using 7.10 and 7.11 we will get that:

7.15. $$Y^{tot} = Y \cdot \left(1 + \sum_{k=1}^{n} \alpha^k\right) + Y_P \cdot \left(1 + \frac{[P]}{K_P}\right)$$

Put another way:

$$7.16. \qquad Y = \left( \frac{Y^{tot}}{1 + \sum_{k=1}^{n} \alpha^{k}} \right) - \left( \frac{1 + \frac{[P]}{K_P}}{1 + \sum_{k=1}^{n} \alpha^{k}} \right) \cdot Y_P$$

$$7.17. \qquad Y_P = \left( \frac{Y^{tot}}{1 + \frac{[P]}{K_P}} \right) - \left( \frac{1 + \sum_{k=1}^{n} \alpha^{k}}{1 + \frac{[P]}{K_P}} \right) \cdot Y$$

Substituting 7.16 and 7.17 in 7.12 and 7.13 will result in:

$$7.18. \qquad \frac{dY}{dt} = \gamma - \eta \cdot Y$$

$$7.19. \qquad \frac{dY_P}{dt} = \gamma_P - \eta_P \cdot Y_P$$

, where the constants are defined as:

$$\gamma \equiv \frac{k_P}{1 + \frac{K_P}{[P]}} \cdot Y^{tot} \quad ; \quad \eta \equiv k_P \cdot \frac{1 + \sum_{k=1}^{n} \alpha^{k}}{1 + \frac{K_P}{[P]}} + a_m^1 \cdot [MAPK^*] - d_m^1 \cdot \alpha^1$$

$$\gamma_P \equiv \frac{k_m \cdot \alpha^n}{1 + \sum_{k=1}^{n} \alpha^{k}} \cdot Y^{tot} \quad ; \quad \eta_P \equiv k_m \cdot \alpha^n \cdot \frac{1 + \frac{[P]}{K_P}}{1 + \sum_{k=1}^{n} \alpha^{k}} + \frac{k_P \cdot [P]}{K_p}$$

Note that constants $\gamma, \gamma_P, \eta$ and $\eta_P$ are functions of the rate constants and the enzyme levels, but are not functions of the Y or $Y_P$. Therefore, the network dynamics is exponential with a time scale of $\eta^{-1}$. Since $\eta$ does not depend on the initial conditions, nor on $Y^{tot}$ levels, over-expression of Yan will not change the network relaxation time. Hence, formulating the network cooperativity as subsequent bindings of $MAPK^*$ molecules will lead to an exponential dynamics of Yan degradation, which is robust to Yan over-expression. A similar conclusion was reached for the cooperative model involving simultaneous binding of several $MAPK^*$ molecules to Yan. Thus, our experimental observation, that the time needed for Yan to reach steady state is significantly longer upon over-expression, is not consistent with either one of the cooperative models.

# 8. Modeling the Kinetics of Yan Degradation Network in the Absence of a Phosphatase

A central assumption of our model for Yan degradation network is that phosphorylation of Yan is reversible. At present, however, there is no experimental indication for the putative phosphatase(s). In this section we will derive a model of Yan degradation network in the absence of a phosphatase ([P] = 0), such that dephosphorylation does not occur. We show in the following discussion that the presence of a phosphatase is crucial for defining a switch-like pattern that resembles our experimental observations. Specifically, we show that in the absence of a phosphatase, non-cooperative models (first and zero order) do not exhibit a switch-like pattern, while the cooperative first order case generates a switch that is insensitive to Yan over-expression. We also show that the existence of the phosphatase is essential for the zero-order ultrasensitivity mechanism.

Without a phosphatase, equations 2.1- 2.6 reduce to:

8.1. $$\frac{dY}{dt} = -a_m Y[MAPK_f^*]^n + d_m[YMAPK^*] + \alpha - \lambda Y$$

8.2. $$\frac{d[YMAPK^*]}{dt} = a_m Y[MAPK_f^*]^n - (d_m + k_m)\cdot[YMAPK^*]$$

8.3. $$\frac{dY_p}{dt} = k_m[YMAPK^*] - \lambda_p Y_p$$

, with one enzyme conservation equation:

8.4. $$[MAPK^*] = n\cdot[YMAPK^*] + [MAPK_f^*]$$

At quasi-steady state equation 8.2 results in:

8.5. $$[YMAPK^*] = \frac{Y\cdot[MAPK_f^*]^n}{(K_m)^n} \quad ; \quad (K_m)^n \equiv \frac{d_m + k_m}{a_m}$$

We will analyze the network for both the non-cooperative case and the cooperative one.

## 8.a. Non-cooperative networks

In the non-cooperative case (n = 1), equations 8.4 and 8.5 reduce to:

8.6. $$[YMAPK^*] = \frac{[MAPK^*]}{1 + \frac{K_m}{Y}} \quad ; \quad [MAPK_f^*] = \frac{[MAPK^*]}{1 + \frac{Y}{K_m}}$$

Substituting 8.6 into 8.1 and 8.3 and assuming steady state, we will get:

8.7. $$0 = -a_m Y \cdot \frac{[MAPK^*]}{1+\frac{Y}{K_m}} + d_m \cdot \frac{[MAPK^*]}{1+\frac{K_m}{Y}} + \alpha - \lambda Y$$

8.8. $$0 = k_m \cdot \frac{[MAPK^*]}{1+\frac{K_m}{Y}} - \lambda_p Y_p$$

Equations 8.7 and 8.8 can be rearranged as follows:

8.9. $$\frac{k_m [MAPK^*]}{1+\frac{K_m}{Y}} = \alpha - \lambda Y$$

8.10. $$Y_p = \frac{k_m \cdot [MAPK^*]}{\lambda_p} \cdot \frac{1}{1+\frac{K_m}{Y}}$$

Equation 8.9 is a quadratic equation for Y. Its solution is:

8.11. $$Y = \frac{-\left(k_m [MAPK^*] - \alpha + \lambda K_m\right) + \sqrt{\left(k_m [MAPK^*] - \alpha + \lambda K_m\right)^2 + 4\lambda\alpha K_m}}{2\lambda}$$

Note that only the (+) solution is relevant since the (-) solution results in a negative Y (since $4\lambda\alpha K_m > 0$). It seems that Y does not exhibit a switch-like behavior in [MAPK$^*$], therefore $Y_p$ and $Y^{tot}$ are also not a sensitive function of the input MAPK$^*$ levels. Note that the non-cooperative solution is valid for both the first and zero order cases.

In order to check whether a network displays a switch-like behavior it is helpful to examine the network's steady state at different limits, and test the transition between those limits.

### 8.a.i. High MAPK$^*$ levels: $k_m [MAPK^*] >> 2\sqrt{\lambda\alpha K_m} + \alpha - \lambda K_m$

Equation 8.11 can be written as:

8.12 $$Y = \frac{\left(k_m [MAPK^*] - \alpha + \lambda K_m\right)}{2\lambda}\left(\sqrt{1+\left(\frac{2\sqrt{\lambda\alpha K_m}}{k_m [MAPK^*] - \alpha + \lambda K_m}\right)^2} - 1\right)$$

Using $\sqrt{1+x} \xrightarrow{x<<1} 1+\frac{1}{2}x$, equation 8.12 reduces to:

8.13. $$Y(high\,[MAPK^*]) = \frac{\alpha}{\lambda + \frac{k_m[MAPK^*] - \alpha}{K_m}}$$

It is clear that in the high MAPK* limit Y is not a sensitive function of the input MAPK* levels.

### 8.a.ii. Low MAPK* levels: $k_m[MAPK^*] << 2\sqrt{\lambda\alpha K_m} + \alpha - \lambda K_m$

In this case, equation 8.11 can be written as:

8.14 $$Y = \frac{-\left(k_m[MAPK^*] - \alpha + \lambda K_m\right) + 2\sqrt{\lambda\alpha K_m} \cdot \sqrt{1 + \left(\frac{k_m[MAPK^*] - \alpha + \lambda K_m}{2\sqrt{\lambda\alpha K_m}}\right)^2}}{2\lambda}$$

Using $\sqrt{1+x} \xrightarrow{x<<1} 1 + \frac{1}{2}x$ and neglecting second order terms, equation 8.12 reduces to:

8.15. $$Y(low\,[MAPK^*]) = \left(\frac{\alpha - \lambda K_m + 2\sqrt{\lambda\alpha K_m}}{2\lambda}\right) - \frac{k_m}{2\lambda} \cdot [MAPK^*]$$

, which linearly depends on MAPK* levels.

### 8.a.iii. Transition between the limits

The transition between this two states (of 'high' and 'low' [MAPK*]) relies on the relation between a term which linearly depends on the input ($k_m[MAPK^*]$) and a constant term ($2\sqrt{\lambda\alpha K_m} + \alpha - \lambda K_m$). Therefore, the transition is relatively 'slow' (Comparing, for example, to the positive feedback case where the threshold location depends on $[MAPK^*]^n$, n >1). Since the two states have low sensitivity to the input MAPK* levels and the transition between them is slow, the network is not expected to exhibit a switch like pattern.

We conclude that without a phosphatase, non-cooperative networks do not exhibit a switch-like behavior. This prediction is not compatible with our experimental observations.

## 8.b. Cooperative networks

In this section we show that cooperative networks do not predict the observed switch-like behavior when no phosphatase is present. We will analyze the first and zero order cooperative networks separately.

### 8.b.i. First order kinetics

At first order ($Y^{tot} << K_m$, $[MAPK^*]$) most of the enzymes are free and therefore 8.4 and 8.5 result in:

8.16. $$[YMAPK^*] \cong \frac{Y \cdot [MAPK^*]^n}{(K_m)^n} \quad ; \quad [MAPK_f^*] \cong [MAPK^*]$$

Substituting 8.16 into 8.1 and 8.3 and assuming steady state, we get:

8.17. $$0 = -a_m Y [MAPK^*]^n + d_m \frac{Y \cdot [MAPK^*]^n}{(K_m)^n} + \alpha - \lambda Y$$

8.18. $$0 = k_m \frac{Y \cdot [MAPK^*]^n}{(K_m)^n} - \lambda_p Y_p$$

Equation 8.17 can be rearranged as follows:

8.19. $$Y = \frac{\alpha}{\lambda + \frac{k_m}{(K_m)^n} \cdot [MAPK^*]^n}$$

Substituting 8.19 into 8.18 we will get:

8.20. $$Y_P = \frac{\alpha}{\lambda_p \cdot \left(1 + \frac{\lambda \cdot (K_m)^n}{k_m [MAPK^*]^n}\right)}$$

Both Y and $Y_p$ are sensitive functions of the input $MAPK^*$ levels. The steady state solution for a cooperative first order network is therefore expected to generate a switch also in the absence of a phosphatase.

The dynamics of Y can be analyzed by substituting 8.16 into 8.1 and 8.3:

8.21. $$\frac{dY}{dt} = \alpha - \left(\frac{k_m [MAPK^*]^n}{(K_m)^n} + \lambda\right) \cdot Y$$

Equation 8.21 shows that Y has exponential dynamics with a time scale $T = \left(\frac{k_m [MAPK^*]^n}{(K_m)^n} + \lambda\right)^{-1}$.

Note that the time scale is independent of the initial conditions and the production rate α. We can conclude therefore that the exponential dynamics in the cooperative case is insensitive to Yan over-expression. A similar conclusion was reached for the cooperative first order model in the presence of a phosphatase. Consequently, the dynamics of cooperative first order networks is

expected to be robust to Yan over-expression regardless of the presence of a phosphatase. This prediction is not consistent with our experimental observations.

### 8.b.ii. Zero order kinetics

At zero order ($Y^{tot} >> K_m$, [MAPK$^*$]) most of the enzymes are in their complex form and therefore 8.4 and 8.5 result in:

8.22. $$[YMAPK^*] \cong [MAPK^*]/n$$

Since: $[MAPK_f^*] = [MAPK^*] - n \cdot [YMAPK^*] = [MAPK^*] - n \cdot \frac{Y \cdot [MAPK_f^*]^n}{(K_m)^n} \longrightarrow 0$ we will get:

8.23. $$[MAPK_f^*] = K_m \cdot \left( \frac{[MAPK^*]}{Y \cdot n} \right)^{\frac{1}{n}}$$

Substituting 8.23 into 8.1 and 8.3 will result in:

8.24. $$\frac{dY}{dt} = -a_m Y \cdot (K_m)^n \cdot \left( \frac{[MAPK^*]}{Y \cdot n} \right) + d_m [MAPK^*]/n + \alpha - \lambda Y$$

8.25. $$\frac{dY_p}{dt} = \frac{k_m [MAPK^*]}{n} - \lambda_p Y_p$$

Equations 8.24 can be rearranged as follows:

8.26. $$\frac{dY}{dt} = \alpha - \frac{k_m [MAPK^*]}{n} - \lambda Y$$

The steady state solution for equations 8.25 and 8.26 is:

8.27. $$Y = \frac{\alpha - \frac{k_m [MAPK^*]}{n}}{\lambda} \quad ; \quad Y_P = \frac{k_m [MAPK^*]}{n \cdot \lambda_P}$$

This solution is valid only when $k_m [MAPK^*] < n \cdot \alpha$. When [MAPK$^*$] levels pass this threshold, all Yan protein that is being generated is immediately phosphorylated such that: $Y = 0$ and $Y_P = Y^{tot} = \frac{\alpha}{\lambda_P}$. This solution shows a graded decrease in Yan levels as MAPK$^*$ levels are increased, and a switch is not generated in this case.

Note that our discussion is significantly different from the case where a phosphatase is present (chapter 4). When a phosphatase is introduced into the zero-order network, an additional

term, $k_p[P]$, is inserted into 8.27 such that a 'two threshold mechanism' is operating. In particular, the solution will be:

8.28. $$Y = \frac{\alpha - \frac{k_m[MAPK^*]}{n} + k_P[P]}{\lambda} \quad ; \quad Y_P = \frac{\frac{k_m[MAPK^*]}{n} - k_P[P]}{\lambda_P}$$

This solution is valid only when: $T_1 \equiv k_P[P] < \frac{k_m[MAPK^*]}{n} < \alpha + k_P[P] \equiv T_2$. When MAPK$^*$ levels pass $T_2$ all Yan is phosphorylated ($Y = 0 ; Y_P = Y^{tot} = \alpha / \lambda_P$), and when MAPK$^*$ levels decrease under $T_1$ all Yan is non-phosphorylated ($Y = Y^{tot} = \alpha / \lambda ; Y_P = 0$). The transition between the two thresholds is expected to be fast, since phosphorylation and dephosphorylation rates are faster than protein production rates ($\alpha << k_p[P]$ and therefore $T_2 - T_1 << T_1$). We therefore conclude that zero-order networks display a switch-like behavior only when phosphatase is present.

The discussion in this chapter shows that the presence of a phosphatase is crucial for defining a switch-like pattern that resembles our experimental observations. We therefore predict that a phosphatase is present, although the postulated phosphatase was not yet found.

# 9. Steady-State Solution for the Yan / Activated MAPK Complex Level at Cooperative First-Order Kinetics

Equation 3.6 for the steady-state of Yan / activated MAPK complex ($[YMAPK^*]$) can be rewritten as:

9.1. $$[YMAPK^*] = \frac{Y[MAPK^*]^n}{(K_m)^n} \cdot \left(1 - \frac{n \cdot [YMAPK^*]}{[MAPK^*]}\right)^n$$

Using the binomial theorem: $(a+b)^n = \sum_{k=0}^{n} \binom{n}{k} a^k b^{n-k}$, first-order kinetics: $[YMAPK^*] \le Y^{tot} << [MAPK^*]$, and taking only high order binomial terms, it can be simplified as:

9.2. $$[YMAPK^*] = \frac{Y[MAPK^*]^n}{(K_m)^n} \cdot \left(1 - n^2 \cdot \frac{[YMAPK^*]}{[MAPK^*]}\right)$$

, and after rearranging:

9.3. $$[YMAPK^*] = \frac{[MAPK^*]}{\frac{(K_m)^n}{[MAPK^*]^{n-1} \cdot Y} + n^2}$$ , where n = $O(1)$

At first-order, Yan level is limiting, such that $Y^{tot} << K_m$. For simplicity, we can also assume that: $Y^{tot} << K_m \cdot \left(\frac{K_m}{[MAPK^*]}\right)^{n-1} \cdot \frac{1}{n^2}$. This condition is fulfilled if we are in "deep" first-order kinetics, such that $K_m$ is very large (e.g. if $O(K_m) = 10 \cdot O([MAPK^*])$). In this case the complex value is:

9.4. $$[YMAPK^*] = Y \cdot \frac{[MAPK^*]^n}{(K_m)^n}$$

## 10. Summary of the Models Parameters and Predictions

The following table summarizes the models parameters and predictions:

| NETWORK KINETICS | | FIRST ORDER | COOPERATIVE FIRST ORDER | FIRST ORDER WITH POSITIVE FEEDBACK | ZERO ORDER |
|---|---|---|---|---|---|
| Network parameters | Positive feedback ($K_f$, l) | $K_f >> Y^{tot}$ | $K_f >> Y^{tot}$ | $K_f << Y^{tot}$ <br> $l > 1$ | $K_f >> Y^{tot}$ |
| | Cooperativity (n) | $n = 1$ | $n > 1$ | $n = 1$ | $n = 1$ |
| | Substrate vs. Enzyme conc. | $Y^{tot} << [MAPK^*],[P]$ | $Y^{tot} << [MAPK^*],[P]$ | $Y^{tot} << [MAPK^*],[P]$ | $Y^{tot} >> [MAPK^*],[P]$ |
| | Substrate vs. Michaelis menten coeff. | $Y^{tot} << K_m, K_p$ | $Y^{tot} << K_m, K_p$ | $Y^{tot} << K_m, K_p$ | $Y^{tot} >> K_m, K_p$ |
| Sensitivity to input values | Is a switch generated? | No | Yes | Yes | Yes |
| Network response to over-expression $\beta > 1$ | Location of Yan degradation border | Changed at least by a factor of $\beta$ | Robust | Changed at least by a factor of $\beta$ | Robust |
| | Time to reach the steady state | Robust | Robust | Increase | Increase dramatically |

**Table 3.** Summary of the models parameters and predictions