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Regulation of oscillation dynamics in biochemical systems with dual negative feedback loops.

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SUMMARY

Feedback controls are central to cellular regulation. Negative feedback mechanisms are well known to underlie oscillatory dynamics. However, the presence of multiple negative feedback mechanisms is common in oscillatory cellular systems, raising intriguing questions of how they cooperate to regulate oscillations. In this work, we studied the dynamical properties of a set of general biochemical motifs with dual, nested negative-feedback structures. We showed analytically and then numerically confirmed that, in these motifs, each negative feedback loop exhibits distinctly different oscillation-controlling functions. The longer, outer feedback loop was found to promote oscillations, whereas the short, inner loop suppresses and can even eliminate oscillations. We found that the position of the inner loop within the coupled motifs affects its repression strength towards oscillatory dynamics. Bifurcation analysis indicated that emergence of oscillations may be a strict parametric requirement and thus evolutionarily tricky. Investigation of the quantitative features of oscillations (i.e. frequency, amplitude, mean value) revealed that coupling negative feedback provides robust tuning of the oscillation dynamics. Finally, we demonstrated that the mitogen-activated protein kinase (MAPK) cascades also display properties seen in the general nested feedback motifs. The findings and implications in this study provide novel understanding of biochemical negative feedback regulation in a mixed wiring context.

1. INTRODUCTION

Feedback regulations are arguably the most common control mechanisms employed by cellular systems, ranging from metabolic to transcriptional to signalling levels (Brandman et al., 2008; Freeman, 2000). Feedback provides cells with self-adjusting capability essential for their survival in fluctuating environments. Failure of proper feedback regulation can lead to diseased states of the cells (Freeman, 2000). A large body of research has thus been dedicated to studying feedback regulation and the implications at cellular levels. While positive feedback is critical in giving rise to multiple stable steady states, underlining switch-like behaviours and prolonging signals (Ferrell, 2002), negative feedback is essential in maintaining homeostasis and generating oscillatory dynamics (Brandman et al., 2008; A. Goldbeter, 1996).

Despite concrete progress, much is still to be understood about the regulation and function of feedback mechanisms in many cellular systems. Advances in molecular techniques are providing better understanding of the mechanistic picture of the cells, which show that feedback mechanisms often appear tangled up and act cooperatively rather than in isolation. Examples of cellular systems in which several negative feedback loops intertwine to coordinate complex dynamics are ubiquitous. At least three different negative feedback mechanisms are found to regulate the oscillatory nucleo-cytoplasmic shuttling of the transcriptional nuclear factor kappaB (Hoffmann et al., 2006). More than two negative feedback loops, acting at different layers of the cascade, were also described in the extracellular signal-regulated kinase (ERK) pathway (Matallan et al., 2011), which may underlie the oscillations recently observed in certain cell types (Cirit et al., 2010; Shankaran et al., 2009). Furthermore, multiple negative feedback structures are prominent in the synthesis pathways of many common amino-acids where the amino acids regulate their own production by concurrently inhibiting multiple upstream reaction steps (Chassagnole et al., 2001; Yanofsky, 2004). For instance, the tryptophan operon has evolved seemingly redundant negative feedback loops but which appear to be providing distinct dynamical and noise-related functions (L.K. Nguyen et al., 2008; L. K. Nguyen, Kulasiri, D, 2010; Santillan et al., 2001). The cooperative feedback structures exemplified above often underline intricate connections between molecular components within cellular pathways as well as crosstalk between them. Breakdown of such concerted operation could lead to malfunctions in the harbouring system and results in diseased state of the cell. Although studies looking at linked feedback structures, e.g. mixed positive and negative feedbacks, have been carried out to some extent, their number remains limited (Pomerening et al., 2003; Tian et al., 2009; Tsai et al., 2008). Structures of mixed negative feedbacks have received far less attention, creating gaps in our understanding concerning their dynamical behaviour.

In the current work, we set out to fill some gaps by studying in detail the dynamical properties of various general dual-negative-feedback motifs with nested structures, using combined analytical and computational approaches. Schematic diagrams of the studied motifs and the single-feedback loop counterpart are given in Figure 1. Each motif consists of two negative feedback loops acting simultaneously at different layers of control. All motifs share the outer feedback loop but differ in the second inner loops, three of them are auto-repression feedback loops. We aimed to understand in these coupled-feedback contexts, how the individual loops contribute to the regulation of oscillations' existence and basic quantitative characteristics including the period (frequency), amplitude and average (mean) value (Fig. S1a). Of particular interest, we asked if the coupled feedback structures provide any enhanced robustness to the control of oscillation. To this end, we obtained analytical conditions based on which parameter regimes leading to distinct dynamical characteristics can be derived. Crossing between these regimes corresponds to qualitative change of the system's behaviours including fixed-point steady state and sustained oscillations, which are the prominent dynamics in the

considered motifs. As a specific example, similar questions were investigated in the well conserved mitogen-activated protein kinase (MAPK) signalling cascades where a nested negative feedback structure is at work (Kolch, 2000; Matallanas et al., 2011).

2. MODELS AND METHODS

2.1. Feedback Motifs and Mathematical Description

Early work on modelling feedbacks in gene-related systems started with the work of Goodwin (Goodwin, 1965). The first repressive model of transcriptional regulation where a gene's expression is inhibited by its own protein product was proposed. This simple model has provided a practical framework for a plethora of subsequent studies exploring the dynamics of negative feedback genetic systems (Bliss et al., 1982; Griffith, 1968; Mallet-Paret et al., 1990; Painter et al., 1981; Sinha et al., 1988; J. Tyson et al., 1978). Here, we extended the Goodwin framework to describe feedback systems with multiple negative mechanisms. A multi-loop system is described by the following differential equations:

$$\begin{aligned} \frac{d[S_1]}{dt} &= k_1 \cdot \prod_{i \in I_1} H(K_{1,i}, n_{1,i}, [S_i]) - k_{d1} \cdot [S_1] \\ \frac{d[S_2]}{dt} &= k_2 \cdot \prod_{i \in I_2} H(K_{2,i}, n_{2,i}, [S_i]) [S_1] - k_{d2} \cdot [S_2] \\ &\dots \\ \frac{d[S_m]}{dt} &= k_m \cdot \prod_{i \in I_m} H(K_{m,i}, n_{m,i}, [S_i]) [S_{m-1}] - k_{dm} \cdot [S_m], \end{aligned} \quad (1)$$

where m is the length of the system, $[S_i]$, k_i and k_{di} ($i = 1..m$) denote concentrations of the molecular species S_i and its rates of synthesis and degradation. Hill kinetics are used to model feedback inhibition (Alon, 2007), given by

$$H(K, n, [S]) = \frac{K^n}{K^n + [S]^n}. \quad (2)$$

In eqn. (1), there is a subindex set I_k ($k = 1..m$) which describes inhibition of S_k via different feedback loops by the species denoted by the subindex k . For instance, $I_1 = \{2, 3\}$ if S_1 is repressed by S_2 and S_3 . Figure 1 displays the scheme of the dual-negative-feedback motifs of length three considered in this study. The ordinary differential equations (ODE) describing their dynamics following the same notations as in eqn. (1) are given in section 1 of the Electronic Supplementary Material (ESM). The input S_0 which can be a gene, ligand or signalling protein that triggers sequential activation of a transcriptional or signalling cascade, was included in parameter k_1 in (1) for convenience. Degradation and synthesis are assumed to follow first-order kinetics. Since the half-saturating constant K is inversely correlated to the inhibiting Hill function, we refer to K as the reciprocal feedback strength of the respective loop (Alon, 2007; Tao et al., 2005). Biologically, K represents the binding constant between the repressor and the repressed target (Tao et al., 2005). A small value of K indicates that the feedback is strong while large K implies weak inhibition. On the other hand, the Hill coefficient n modulates the degree of function sigmoidity and signifies the level of nonlinearity exhibited by the feedback loop.

2.2. Analytical Analysis of Dynamics Based on the Routh-Hurwitz Theorem

Biochemical systems can exhibit diverse types of dynamical behaviour including fixed point, mono- or bi-stable steady states, oscillations (limit cycles) and even irregular fluctuation (chaos) (Kholodenko et al., 2010; Mestl et al., 1996). Sustained oscillations and fixed-point steady states are often the prominent dynamics displayed by negative feedback systems which are separated by Hopf bifurcation (Mallet-Paret et al., 1990; J. Tyson et al., 1978). Four motifs in Fig.1(a,d-f) can be categorised as monotone cyclic negative feedback systems and thus have been proved to only exhibit fixed point or sustained oscillations (Mallet-Paret et al., 1990). Linear stability analysis hence provides a convenient way to detect oscillations in these systems. For the remaining dual-feedback motifs which are non-monotone cyclic (Fig.1b-c), further methods are required to validate oscillations, which we will discuss below. We focus here on how the transition between these two dynamics is governed for the considered motifs.

Dynamical insights of a system can be obtained by linearising the governing differential equations around the equilibrium state and probing the eigenvalues of the resulting Jacobian matrix, J , given by (Murray, 2002):

$$J = \begin{pmatrix} \frac{\partial f_1}{\partial x_1} & \dots & \frac{\partial f_1}{\partial x_n} \\ \vdots & \ddots & \vdots \\ \frac{\partial f_n}{\partial x_1} & \dots & \frac{\partial f_n}{\partial x_n} \end{pmatrix}, \text{ where } \begin{cases} x'_1 = f_1(x_1, x_2, \dots, x_n) \\ \vdots \\ x'_n = f_n(x_1, x_2, \dots, x_n) \end{cases} \quad (3)$$

If the real parts of all J 's eigenvalues are negative, the system's equilibrium is deemed stable and reaches a fixed-point steady state, whereas if any of the real parts are positive, system stability is lost and in the motifs considered here, the system moves into a limit cycle regime where it oscillates. The eigenvalues are usually obtained numerically and their real part values are assessed. To facilitate analytical treatment, we instead employ the Routh-Hurwitz theorem (Gradshteyn et al., 2000; Murray, 2002) which states that the eigenvalues, essentially the roots of the characteristic polynomial

$|J - \lambda I| = \lambda^n + \alpha_1 \lambda^{n-1} + \dots + \alpha_{n-1} \lambda + \alpha_n = 0$, all possess negative real parts if and only if the following condition holds true

$$\Delta_1 > 0, \Delta_2 > 0, \dots, \Delta_n > 0, \quad (4)$$

where Δ_k ($k = 1..n$) are the determinants of the properly constructed matrices

$$\Delta_k = \begin{vmatrix} \alpha_1 & 1 & 0 & 0 & 0 & 0 & \dots & 0 \\ \alpha_3 & \alpha_2 & \alpha_1 & 1 & 0 & 0 & \dots & 0 \\ \alpha_5 & \alpha_4 & \alpha_3 & \alpha_2 & \alpha_1 & 1 & \dots & 0 \\ \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \ddots & \vdots \\ \alpha_{2k-1} & \alpha_{2k-2} & \alpha_{2k-3} & \alpha_{2k-4} & \alpha_{2k-5} & \alpha_{2k-6} & \dots & \alpha_k \end{vmatrix}$$

Eqn. (4) therefore represents the sufficient condition for oscillations in the dual-feedback motifs while it is only a necessary condition in motifs $F_{31,32}$ and $F_{31,21}$. To verify that (4) also constitute the sufficient condition for oscillations in these systems, we employed analysis based on the Floquet theory that globally assesses the stability of the obtained periodic solutions (Apri et al., 2010; Klausmeier, 2008; Nayfeh et al., 1979). The mathematical and computational background of the Floquet analysis is given in the ESM. This method was numerically implemented in Mathematica 8.0 (code can be provided on request). In the following sections, we will present first the analytical analysis based on (4) for the single-loop system F_{31} followed by the exemplified dual-loop motif $F_{31,32}$. Detailed derivations for the remaining motifs are provided in the ESM.

3. RESULTS

3.1. Dynamical Analysis of the Single-loop System F_{31}

We begin by examining the single negative feedback motif F_{31} , where the first species in the cascade S_1 is inhibited by the last species S_3 . The ODE model of this motif is given in section 1, ESM. Systems of this type have been previously studied assuming equal degradation rates (J. Tyson et al., 1978; Zeiser et al., 2007), yielding informative results but are limited in their biological implication due to the oversimplified assumption. Our following analysis assumes no such restricting assumption and was aimed to facilitate an extension to motifs of multiple feedback loops. According to the Routh-Hurwitz theorem, F_{31} reaches the steady state if and only if

$$\begin{cases} \alpha_1 > 0, \alpha_2 > 0 \\ \alpha_1 \alpha_2 > \alpha_3 \end{cases} \quad (5)$$

where $\alpha_1, \alpha_2, \alpha_3$ are the coefficients of the system's characteristic polynomial $f(\lambda) = \lambda^3 + \alpha_1 \lambda^2 + \alpha_2 \lambda + \alpha_3$.

Hereafter, we denote x_i as the steady-state values (when the system reaches equilibrium) of S_i ($i = 1, 2, 3$).

These steady-state variables of the system can be obtained by setting the right hand sides (RHS) of eqn. (1) to zero which leads to

$$k_1 \frac{K_1^{n_1}}{K_1^{n_1} + x_3^{n_1}} = k_{d1} x_1, \quad k_2 x_1 = k_{d2} x_2 \quad \text{and} \quad k_3 x_2 = k_{d3} x_3 \quad (6)$$

$$\text{This yields } K_1^{n_1} / (K_1^{n_1} + x_3^{n_1}) = f \cdot x_3, \quad \text{where } f = \frac{k_{d1} k_{d2} k_{d3}}{k_1 k_2 k_3} \quad (7)$$

Using eqn. (6), the coefficients $\alpha_1, \alpha_2, \alpha_3$ are given by

$$\alpha_1 = k_{d1} + k_{d2} + k_{d3}, \quad \alpha_2 = k_{d1} k_{d2} + k_{d1} k_{d3} + k_{d2} k_{d3}, \quad \alpha_3 = k_{d1} k_{d2} k_{d3} (1 + n_1 M_1),$$

$$\text{where } M_1 = x_3^{n_1} / (K_1^{n_1} + x_3^{n_1}). \quad (8)$$

This introduction of a composite variable M_1 ($0 < M_1 < 1$), as will soon become apparent, allows us to ignore possible complications involving x_3 and the related rational functions such as those in eqns. (6) and (7), which greatly facilitates our treatment. Since $\alpha_1, \alpha_2 > 0$, eqn. (5) means that oscillatory dynamics arise if $\alpha_1 \alpha_2 - \alpha_3 < 0$. Substituting $\alpha_1, \alpha_2, \alpha_3$ into this condition gives

$$M_1 > \frac{g}{n_1}, \quad \text{where } g = (k_{d1} + k_{d2})(k_{d2} + k_{d3})(k_{d1} + k_{d3}) / (k_{d1} k_{d2} k_{d3}). \quad (9)$$

Rearranging x_3 in terms of M_1, K_1 and n_1 from eqn. (8) and substitute x_3 into eqn. (7), we have:

$$1 - M_1 = f \cdot x_3 = f \cdot K_1 \cdot \sqrt[n_1]{\frac{M_1}{1 - M_1}}.$$

K_1 can now be expressed as $K_1 = F(M_1)$, where $F(M_1) = \frac{1 - M_1}{f} \sqrt[n_1]{\frac{1}{M_1} - 1}$ is a function of M_1 .

Because $dF(M_1)/dM_1 < 0$ for $M_1 \in [0, 1]$, $F(M_1)$ strictly decreases with M_1 , the oscillations condition, eqn. (9), is transformed into the following equivalent form

$$K_1 < F\left(\frac{g}{n_1}\right) = \frac{n_1 - g}{n_1 f} \sqrt[n_1]{\frac{1}{g} - 1}, \quad (10)$$

where f and g involve the synthesis and degradation parameters given in eqns. (7) and (9).

The symbolic, parametric condition eqn. (10) governs the bifurcation points separating the dynamics of the fixed-point steady state and limit cycles of the single-loop system. It is immediately clear that the system can only oscillate if the feedback loop is sufficiently strong (if K_1 small enough). Moreover, the Hill coefficient n_1 must be greater than g for any chance of oscillations to realise. The latter observation is consistent with the previous derivation by Griffith (1968).

3.2. Dynamical Analysis of the Dual-feedback Systems

Demonstrating this, we describe here treatment of the dual-feedback system $F_{31,32}$, while only the main results are reported for other motifs whose detailed derivations are given in the ESM. Motif $F_{31,32}$ contains a second, inner feedback loop where the cascade product S_3 also inhibits species S_2 . The governing ODEs are presented in section 1, ESM. This newly incorporated loop is also modelled by Hill kinetic function in which the inverse of the parameter K_2 describes the inhibition strength, and n_2 measures the nonlinearity level of the feedback regulation. The steady-state solution of the system variables can be obtained by setting the RHS of the differential equations to zero which leads to

$$k_1 \frac{K_1^{n_1}}{K_1^{n_1} + x_3^{n_1}} = k_{d1} x_1, \quad k_2 \frac{K_2^{n_2}}{K_2^{n_2} + x_3^{n_2}} x_1 = k_{d2} x_2 \quad \text{and} \quad k_3 x_2 = k_{d3} x_3 \quad (11)$$

Introducing $M_1 = x_3^{n_1} / (K_1^{n_1} + x_3^{n_1})$ and $M_2 = x_3^{n_2} / (K_2^{n_2} + x_3^{n_2})$,

$$\text{from eqn. (11) we obtain } (1 - M_1)(1 - M_2) = f \cdot x_3, \quad (12)$$

where f is given in eqn. (7). The coefficient $\alpha_1, \alpha_2, \alpha_3$ of the characteristic polynomial in this case can be expressed as

$$\alpha_1 = k_{d1} + k_{d2} + k_{d3}, \quad \alpha_2 = k_{d1} k_{d2} + k_{d1} k_{d3} + k_{d2} k_{d3} (1 + n_2 M_2)$$

$$\alpha_3 = k_{d1} k_{d2} k_{d3} (1 + n_1 M_1 + n_2 M_2),$$

$$\text{The oscillating condition } \alpha_1 \alpha_2 - \alpha_3 < 0 \text{ now becomes } M_1 > \alpha + \beta M_2, \quad (13)$$

where $\alpha = g/n_1, \beta = (n_2/n_1)(k_{d2} + k_{d3})/k_{d1}$.

Since x_3 can be written as $x_3 = K_1 \cdot \sqrt[n_1]{M_1/(1 - M_1)}$, substitute this into eqn. (12) and rearrangement yields

$$K_1 = \frac{1 - M_1}{f} \cdot \sqrt[n_1]{\frac{1 - M_1}{M_1}} \cdot (1 - M_2) \Leftrightarrow K_1 = F(M_1)(1 - M_2) \quad (14)$$

where $F(M_1)$ was previously defined. Since $F(M_1)$ strictly decreases with $M_1 \in [0, 1]$, eqn. (14) becomes

$$K_1 < F(\alpha + \beta M_2)(1 - M_2) \quad (15)$$

It is convenient here to report similar conditions obtained for the remaining dual-feedback motifs (see ESM for details).

$$\text{For } F_{31,33}: K_1 < F(\alpha + \beta M_2 + \gamma M_2^2)(1 - M_2), \quad (16)$$

where $\alpha = g/n_1, \beta = (n_2/n_1)(k_{d1} + k_{d2})(k_{d1} + k_{d2} + 2k_{d3})/k_{d1}k_{d2}$ and $\gamma = (n_2^2/n_1)(k_{d1} + k_{d2})k_{d3}/k_{d1}k_{d2}$.

$$\text{For } F_{31,21}: K_1 < F(\alpha + \beta M_2)(1 - M_2), \quad (17)$$

where $\alpha = g/n_1, \beta = (n_2/n_1)(k_{d1} + k_{d2})/k_{d3}$.

$$\text{For } F_{31,11}: K_1 < F(\alpha + \beta M_2 + \gamma M_2^2)(1 - M_2), \quad (18)$$

where $\alpha = g/n_1, \beta = (n_2/n_1)(k_{d2} + k_{d3})(k_{d2} + k_{d3} + 2k_{d1})/k_{d2}k_{d3}$ and $\gamma = (n_2^2/n_1)(k_{d3} + k_{d2})k_{d1}/k_{d2}k_{d3}$.

$$\text{For } F_{31,22}: K_1 < F(\alpha + \beta M_2)(1 - M_2), \quad (19)$$

where $\alpha = g/n_1$, $\beta = (n_2/n_1)(k_{d1} + k_{d3})(k_{d1} + k_{d3} + 2k_{d2})/k_{d1}k_{d3}$ and $\gamma = (n_2^2/n_1)(k_{d1} + k_{d3})k_{d2}/k_{d1}k_{d3}$.

3.3. Addition of the Inner Feedback Loops Reduces and Potentially Eliminates Oscillatory Dynamics

It can be observed that eqns. (15-19) share a common form despite the difference in the coefficients of the M_2 -related terms inside function F . Moreover, an extra term $(1 - M_2)$ appears on the RHS of these equations that does not appear in (10) derived earlier for F_{31} . It turned out that this additional term and those inside the function F brings about important implications in how the inner negative feedback loops affect system dynamics in the dual-feedback motifs, as shown below.

First, since the composite variable M_2 belongs to the $[0,1]$ interval, the following holds true for all dual-feedback motifs:

$$\begin{aligned} K_1 &= F(\alpha + \beta M_2 + \gamma M_2^2)(1 - M_2) \\ &< F(\alpha)(1 - M_2) < F(\alpha) = F\left(\frac{g}{n_1}\right) \end{aligned} \quad (20)$$

Note that the form of β are motif specific and $\gamma = 0$ for $F_{31,21}$ and $F_{31,32}$. Eqn. (20) indicates that $K_1 < F(g/n_1)$ is a necessary condition for oscillations in the dual-feedback motifs which, in comparison to condition eqn. (10), implies that addition of any of the negative feedback loops: S_3 repressing S_2 , S_2 repressing S_1 , or auto-repression of S_1 , S_2 or S_3 would not produce oscillations if the F_{31} does not oscillate in the first place. Eqn. (20) also suggests that the dual-feedback structures reduce the effective upper-bound threshold of K_1 possible for oscillations, thereby shrinking the oscillations domain compared to the single-loop motif.

Secondly, consider the case when the second feedback loop is weak. In this scenario, K_2 is large which makes $M_2 \approx 0$. The RHS of eqns. (15-19) then become $F(\alpha)$. The condition for oscillations in the coupled-loop motif $F_{31,32}$ thus approaches that of the single-loop motif F_{31} in the limiting case of weak feedback inhibition by the inner loop. On the other hand, when the inner loop is strong, K_2 is large resulting in $M_2 \approx 1$. The RHS of eqns. (15-19) then become null which would lead to unsatisfactory conditions for oscillations if $K_1 > 0$. An important and rather unexpected consequence is that the inner feedback loop, when sufficiently strong could completely eliminate oscillations by overriding the oscillatory dynamics triggered by the outer feedback loop. To verify these predictions, we performed numerical simulations of all the motifs with arbitrary parameter sets, which indeed showed that pre-existing oscillations in the single-loop system F_{31} can be abolished when incorporating a sufficiently strong second feedback loops; whereas oscillations are maintained for weak inhibition levels (Fig. S2).

3.4. Dynamics Partitioning Revealed Differential Oscillations-Reducing Efficiency by the Dual-feedback Motifs

Although eqns. (15-19) allowed us to draw novel insights into the effects of the nested feedback structures on the appearance likelihood of oscillations, the presence of M_2 in these equations complicates effort to derive conditions involving only the model parameters. Below, we further refine these equations to achieve this very aim.

Again, we use the system $F_{31,32}$ as an illustrative case. Since x_3 can also be written as $x_3 = K_2 \cdot \sqrt[n_2]{M_2/(1 - M_2)}$, eqn. (12) enables us to express M_1 in terms of M_2 as follows

$$\begin{aligned} M_1 &= 1 - f \cdot x_3 / (1 - M_2) = 1 - f \cdot K_2 \cdot \sqrt[n_2]{M_2/(1 - M_2)} / (1 - M_2) \\ &= 1 - K_2 / F_1(M_2) \end{aligned} \quad (21)$$

where $F_1(M_2) = f^{-1}(1 - M_2) \sqrt[n_2]{(1 - M_2)/M_2}$ is a function of M_2 . Note that function F_1 is similar to F defined in section 3.1 except for the parameter n_2 . Eqn. (14) can then be rewritten as

$$K_1 = F_2(M_2), \quad (22)$$

where $F_2(M_2) = F(1 - K_2 / F_1(M_2))(1 - M_2)$.

Since $F_2(M_2)$ strictly increases with M_2 due to $dF_2(M_2)/dM_2 > 0$ (for $M_2 \in [0,1]$ (see the ESM), derivation of the parameters-only condition that governs oscillatory dynamics for the considered motif is simplified to determination of values of the variable M_2 satisfying eqn. (14) or the equivalent form

$$G(M_2) > \alpha + \beta M_2, \quad (23).$$

where $G(M_2) = 1 - K_2 / F_1(M_2)$.

Such ranges of M_2 cannot be obtained analytically but can be quickly obtained by numerically solving eqn. (23), and can be conveniently visualised on the M_1 vs. M_2 coordinate where intersection of the functions

$G(M_2)$ and $\alpha+\beta M_2$ are shown (Fig. 3a). Plugging the obtained numerical value of M_2 into eqn. (22) allows us to readily compute the value of K_1 that result in oscillations given any parameterization of the remaining model parameters. Bifurcation plots which partition the parameter space (i.e. K_1 versus other parameters) can therefore be constructed and based on these, we will be able to perceive how changes in certain parameters affect system dynamics and bring about oscillations. It should be noted that the form of the function F_2 is slightly different across the motifs, as detailed in the ESM.

Figure 3b compares the partition of systems dynamics (fixed-point steady state and oscillations) on the K_1 vs. K_2 coordinate for all five dual-negative-feedback motifs. Consistently in all these motifs, a second loop with strong inhibition, signified by small K_2 , tends to bring the system out of the oscillation domain. In fact, we observed an increasing effect where stronger second loops result in smaller size of the oscillation domain. Since the same value of n_2 was used for all motifs in Figure 3b, it reveals that the ability to diminish oscillations is weakened as the second loop moves upstream of the cascade. Indeed, addition of S_3 's auto-inhibition loop gives the smallest oscillations domain, whereas it is largest with auto-inhibition of S_1 . Furthermore, the figure shows, as predicted, that K_1 approaches a threshold value ($F(g/n_1)$) at large K_2 , which is independent of n_2 .

3.5. Dependence of Oscillations on the Degradation Rates and Optimisation of Oscillation's Appearance

The rates of degradation have been suggested to play important roles in influencing the existence of oscillations in feedback systems (Forger, 2010; Gerard et al., 2009; Ruoff et al., 1999). Symmetry among elements within a feedback loop was predicted to increase the occurrence likelihood of oscillations (Forger, 2010). It is therefore interesting to study how the degradation rates affect systems dynamics in our dual-feedback context. Figure 4 shows on the K_1 vs. k_{di} bifurcation diagrams ($i=1..3$) that the threshold K_1 depends biphasically on the degradation rates in all motifs. The bell-shaped oscillation domains suggest that oscillatory behaviour arises only over a bounded range of the degradation rates. The three-dimensional bifurcation plot (Fig. 4d) of K_1 in response to simultaneous changes in pairs of the degradation rates further shows a cone-shaped oscillation domain. The oscillation domain of all the motifs projected in the space of the degradation rates would therefore form a restricted volume, whose size is expectedly diminished as the second feedback loop's inhibition intensity increases. The ability to oscillate is only realised when the rates of degradation are tuned within this enclosed domain, conferring oscillatory dynamics much less probable in the considered motifs compared to steady state equilibrium dynamics. The biphasic dependence of the threshold K_1 on the rates of degradation also implies an optimal strength of the S_3 -repressing- S_1 feedback inhibition at which the appearance of systems oscillations is maximised. Importantly, this optimal state is achieved at intermediate levels of the turnover rates of the components within the loop.

Next, we ask the question, what distribution of the degradation rates would lead to maximal oscillations given a fixed steady-state level of the output S_3 ? This comparative criterion is realistic since cellular pathways typically maintain a functional range of key proteins. For the single-loop motif F_{31} , constant x_3 can be brought about by fixing the product of the rates of degradation (see eqn.(7)). We found that in this case, oscillations are most likely under conditions of equal degradation rates, which agrees with previous prediction (Forger, 2010) (see proof in section 2.1, ESM). For the dual-feedback systems $F_{31,32}$ and $F_{31,33}$, constant steady-state S_3 levels can be similarly guaranteed by fixing the product $k_{d1} k_{d2} k_{d3}$. We proved analytically that oscillations are most likely for $F_{31,32}$ when $k_{d2} = k_{d3}$ at any given k_{d1} . On the other hand, $F_{31,33}$ is most prone to oscillations when $k_{d1} = k_{d2}$ at a given k_{d3} (section 2.2, 2.3, ESM). To verify these conclusions, e.g. for $S_{31,32}$, we generated numerous parameter sets that differ only in k_{d2} , k_{d3} but keep their product fixed. The corresponding value of the threshold K_1 was then computed for each set (Fig. S2c, ESM) which indeed shows that the threshold K_1 is maximised at comparable levels of k_{d2} and k_{d3} . Interestingly, similar analysis shows that K_1 is also maximised at comparable $\{k_{d1}, k_{d2}\}$ (Fig. S2a) and $\{k_{d1}, k_{d3}\}$ (Fig. S2b), suggesting that oscillations in $F_{31,32}$ are most probable under equal degradation rates of all species ($k_{d1} = k_{d2} = k_{d3}$). This is also the case for the system $F_{31,33}$, as shown in Figure S2d-f, ESM. For the dual-feedback motifs $F_{31,21}$, $F_{31,22}$ and $F_{31,11}$, constant steady-state S_3 is brought about either by fixing $\{k_{d3}, k_{d1}k_{d2}\}$, $\{k_{d3}, k_{d1}k_{d2}\}$ or $\{k_{d1}, k_{d2}k_{d3}\}$. We found that oscillations are most likely for $F_{31,21}$, $F_{31,22}$ and $F_{31,11}$ when $k_{d1} = k_{d2}$, $k_{d1} = k_{d2}$ and $k_{d2} = k_{d3}$. Analytical proofs of these predictions are shown in section 2.4 and 2.5 of the ESM, and also confirmed by computational simulations (Fig. 5 and S3). These results suggest that in systems with nested feedback structure, symmetry among the elements of the loops also promotes systems oscillations.

3.6. Higher Nonlinearity of Both Feedback Loops Enhances Oscillations.

Negative feedback regulation requires a sufficient degree of nonlinearity for oscillations to arise (Novak et al., 2008). This is evident from the condition $n_1 \geq g$ needed for oscillations in the single-loop and in the tested dual-feedback systems. Since in the dual-feedback motifs, the feedback strength of the individual loops brought about opposing effects on systems oscillations, we asked if the loops' nonlinearity levels behave in the same way. Figure 5 shows that this is not the case. In all the tested dual-feedback motifs, increasing the Hill coefficients n_1 and n_2 both enlarged the oscillations domain projected here on the K_1 vs. K_2 coordinate, leading to enhanced oscillations. However, the underlying mechanisms of this enhancement are different. While higher n_1 supports oscillations by raising the threshold K_1 , allowing oscillations possible at an even weaker S_3 -repressing- S_1 loop strength, higher n_2 enables oscillations at even stronger intensity of the inner loops. Taken together, the result implies that although addition of stronger inner loops diminished oscillations, raising its nonlinearity level tolerated this effect and thus promoted oscillations instead.

3.7. Regulation of quantitative properties of oscillation dynamics in the dual-feedback structures.

An oscillatory dynamic bears a number of basic quantitative characteristics such as its frequency (period), amplitude (difference between peak and trough) and the average value (mean) (Fig. S1a). Recent studies report that these metrics may play important roles in directing cellular responses. In yeast, the level of extracellular calcium was found to modulate the nuclear translocation frequency of transcriptional factor Crz1 and this frequency directly controls expression of multiple target genes (Cai et al., 2008). In calcium and endocrine oscillations, the frequency was also thought to contain key functional signal (Bootman et al., 1996; A. Goldbeter, 1996; A. Goldbeter et al., 1990). In other cases, the amplitude plays key physiological roles. Allada et al. (2003) showed that a clock gene mutant in *Drosophila melanogaster* which produces persistent oscillations but with reduced amplitude led to behavioural malfunction. Previous computational work on mixed positive-negative feedback oscillators revealed such coupling enabled robust tuning of the frequency at stable amplitude (Tsai et al., 2008). It is therefore of our interest to investigate how the three metrics (period, amplitude, mean) of the oscillations dynamics are controlled in the mixed negative-feedback motifs.

First, to understand how the individual loops affect the oscillation pattern in the dual-feedback motifs, the feedback strength of either loop was varied and the period, amplitude and mean value of the oscillatory level of the species S_3 were computed (see section 4.1, ESM for computational procedure). The parameter values used in our calculation was based on the physiological values obtained for the endogenous circadian clock system in *Neurospora crassa* (Ruoff et al., 1999). The molecular basis of this system is described in Figure S1b which follows the general motif F_{31} . Our computational result showed that the period increases with stronger feedback of the outer loop while decreases towards zero (i.e. loss of oscillations) at higher feedback of the nested loop (Fig. 6a) This was observed for all the dual-feedback motifs which is consistent with our previous finding that the second feedback loops can eliminate oscillations when sufficiently strong. We observed a biphasic dependence of the amplitude on the outer loop, indicating existence of an optimal strength a maximal amplitude (Fig. 6b). On the other hand, the inner loops decrease the amplitude across all motifs as expected. Also as expected, both loops decrease the average value of the oscillatory S_3 concentration (Fig. 6c).

Next, we simultaneously varied the strength levels of both the outer and inner loops and recorded the period, amplitude and mean value. Plotting one metric against another in parametric planes revealed interesting observations (Fig. 6d-f). We found that in all the dual-feedback motifs, altering the short inner loop allowed the system to widely modulate the period or amplitude while keeping the mean value at near-constant levels, indicated by the almost perfect horizontal lines in Fig. 6e, f. On the other hand, varying the long outer loop allowed the systems to modulate the period while keeping the amplitude relatively stable. This effect is increased when the systems are well within the oscillating regime, indicated by the lines inside the box in Fig. 6d. Furthermore, higher inner loop seemed to make the tunability more pronounced (lower lines, Fig. 6d). These observations suggest that having coupled negative feedbacks provides the systems with robust ways to adjust different features of the oscillation dynamics, an ability similar to that of the mixed positive-negative motifs (Tsai et al., 2008). Such robustness may be the key in enabling the systems to decouple functional controls regulated by different features of the oscillation dynamics. To see to what extent the above findings are dependent on parameterization, we carried out similar analysis for other random parameter sets generated from the basal set (Fig. S5a and section 3, ESM). We found that the findings still hold true for these sets, suggesting that they are likely consequences of the motifs' topology.

3.8. The MAPK signalling cascade: an example

The MAPK signalling cascades are among the best studied signalling systems in cells, which play crucial roles in many cellular processes including proliferation and survival (Kolch, 2000; Matallanas et al., 2011). Kholodenko (2000) predicted that sustained oscillations can occur in these cascades under physiological parametric conditions. This prediction has been backed up by recent experimental reports (Nakayama et al., 2008; Shankaran et al., 2009). The MAPK cascade model analysed by Kholodenko (2000) however contained only a single negative feedback loop caused by inhibition of the active form of the upstream GTPase (e.g. RasGTP) by the activated MAPK protein, while in fact multiple negative feedback loops are at work (Matallanas et al., 2011). To further understand oscillation dynamics of the MAPK cascades in multi-loop context, we analysed an extended MAPK model. We considered an additional negative feedback brought about by inhibition of the MAPK kinase kinase (MKK) by the active MAPK protein (ppMAPK, Fig. 7a). For instance in the extracellular signal-regulated kinases (ERK) pathway, an exemplary MAPK cascade, the second negative feedback is caused by phosphorylation of Raf-1 on inhibitory sites by active ERK (Kolch, 2000; Matallanas et al., 2011).

The ODE and parameter values of the extended Kholodenko model are given in Tables S1-2, ESM. The feedback strength of the two loops can be adjusted by varying parameters K_{11} and K_{12} . Our numerical stability analysis showed that these feedback loops affect oscillations differently (Fig. 7b). Higher strength of the outer loop tends to move the system into the oscillatory domain, thereby enhancing oscillations, while a stronger inner loop tends to move the system out of the oscillatory domain, thereby weakening oscillations (Fig. 7b). Similar to the general dual-feedback motifs, a sufficiently strong inner loop can destroy oscillations in the MAPK cascades. However, unlike in the general motifs where the outer loop can always rescue oscillations, in the MAPK cascades there exists a barrier strength of the inner loop over which the system fails to oscillate regardless of how strong the outer loop is. This difference may be due to the different wiring and level of

complexity in the MAPK model. We further computed the period, amplitude and mean value of the oscillatory ppMAPK levels in response to increasing strength of the outer and inner loop (Fig. S6). Interestingly, both loops reduced the oscillations period and mean value. The outer loop also exhibited a bi-phasic modulation of the amplitude as expected, while the inner loop consistently decreased the amplitude.

4. DISCUSSION

Understanding the emergence of functional behaviour from the dynamical controls of cellular networks is of fundamental importance in biology. It is therefore critical to dissect how certain system dynamics are brought about and how they are regulated. Oscillations are ubiquitously observed in cells and have been shown to play important roles in a broad array of cellular processes (Hess, 2000; Paszek et al., 2010). The oscillatory behaviour produced can be time-dependent, as in glycolytic oscillations (Ghosh et al., 1964), the circadian rhythms (Panda et al., 2002), the cell cycles (J. J. Tyson et al., 2001), or space-dependent, as in calcium waves (Bootman et al., 2001; Rottingen et al., 2000) and neuronal oscillations (Rabinovich et al., 1998). In all of these examples, oscillations are driven by some sort of negative feedback mechanism. However, in many oscillating systems, their circuits contain more than one negative feedback loop which begs the question how they might cooperate to control oscillations. In the current work, we studied the emergence and control of oscillation dynamics in the context of coupled, nested negative feedback biochemical motifs. By comparative examination of the dual-loop and single-loop structures we aimed to dissect the relevant roles of the individual feedback loops. To this end, we employed combined analytical and numerical methods.

Most interestingly, we found that the individual feedback loops regulate oscillations in opposing manners in all of the general dual-feedback motifs. The long, outer loop promotes oscillations whereas the short, inner loops strictly inhibit oscillations. Such oscillations-inhibiting effects, when sufficiently strong, can dominate the oscillation-generating effect of the outer loop and completely destroy oscillations. Coupling multiple negative feedbacks together thus can suppress rather than enhance oscillations, as found previously (Kim et al., 2008). Note that other ways of negative feedback coupling such as mutual inhibition can also inhibit oscillations and promote bistability instead (Ferrell, 2002), as they behave like a positive feedback. Such wiring is however structurally different to the nested feedback motifs considered here, highlighting that different ways of coupling may yield similar system responses yet through distinct mechanisms. Our analytical proof further showed that the above observation is parameter independent and rather a feature of the network topology. Comparison of the oscillations domain among the dual-feedback motifs under the same parametric specification of the inner feedback loop revealed that as it moves further downstream of the cascade, it represses oscillations more strongly. Auto-inhibition of the final component of the cascade therefore reduces oscillations most efficiently.

Having showed that the shorter loop of the dual-feedback structures can abolish oscillations driven by the longer loop, it is useful to understand this in an intuitive way. Negative feedback requires sufficient delay and nonlinearity to give rise to oscillations (Novak et al., 2008). Our simulations show that removing the outer loop from the dual-feedback motifs rendered them incapable of exhibiting oscillations regardless of parameter choice (data not shown). The failure of these short loops to maintain oscillations on their own may be due to the lack of delay, a cause which is likely to also underline their ability to suppress oscillations in the coupled structures. To test this idea, we constructed variant motifs of the system $F_{31,11}$ which extend the auto-inhibition loop of S_1 by one or two intermediate components (Fig.S1c-d). Addition of one intermediate component still reduced oscillations; while having two intermediates introduced enough delay to prevent the extended loop from having an oscillations-reducing property. One important consequence then follows regarding the modelling of feedback systems in general. Models of biochemical systems are in many cases greatly simplified. Quite often, multiple activation steps occurring via intermediate components are described as a single step for convenience without explicitly taking into account the time delay. We, however, showed that description of a negative feedback with one or more intermediate components could result in markedly different effects of the loop towards system dynamics. Time delay, if significant, thus should be considered in the model for faithful representation of the system in reality.

It also follows from our results that motifs formed by combining the outer feedback loop and any combination of the secondary inner loops would behave similarly to the dual-feedback motifs in their ability to suppress oscillations. The opposing roles of the coupled feedbacks suggest intriguing implications regarding the evolution of negative feedback structures. In principle, changes in system dynamics such as loss of oscillatory behaviour can be brought about by mutations that break the oscillation-generating feedback loop, or alternatively by emergence of new feedback loops with oscillation-abolishing property. Under certain scenarios, the latter strategy may be more advantageous than the former, for example when the newly evolved loops provide additional benefit to the cells. Since measurements like period, amplitude and mean value are intrinsic features of an oscillatory dynamics, we went on to examine how these metrics are regulated by the feedbacks interplay in the linked-feedback motifs. We found that the inner loop enabled flexible modulation of the period or amplitude while keeping the mean value at near-constant levels. More interestingly, in presence of strong inner loop, varying the outer loop allowed the systems to significantly change the period while keeping the amplitude relatively stable. Such features were not possible in the single-loop motif. Our findings suggest that having nested feedback structures provides robust controls of oscillation dynamics and its basic quantitative characteristics. Such robustness may have underlined the evolution of some biochemical oscillators possessing these topological structures. To further our investigation in a more biologically realistic

system, we asked similar questions in the MAPK signalling cascades, which was known to regulate important cellular processes and contain multiple negative feedback loops in a nested structure. To this end, we extended an existing mathematical model for the dual-feedback MAPK cascade. Our analysis showed that despite the fact that the MAPK model is much more intricate, its negative feedbacks also exhibit opposing regulation of oscillations as in the general motifs. It would be interesting to test this prediction experimentally in the future.

Parameter sensitivity analysis is of great importance in understanding biological networks. The derivation of the analytical conditions that govern the occurrence of oscillations have allowed us to construct useful bifurcation plots based on accurate knowledge of when switching between different dynamical regimes occurs. Such knowledge is potentially useful in experimental design. It can also aid in the engineering of synthetic circuits with desirable targeted dynamical behaviour, since one could effectively choose appropriate points for perturbation to attain desired dynamics. In particular, we observed that oscillations only occur over limited ranges of degradation rates. The fact that each of these rates must be tuned within its viable range for oscillations to occur suggests that oscillatory behaviour is a strict parametric requirement. Its emergence cannot be guaranteed by the presence of negative feedback alone but depends greatly on the evolutionary tuning of the stability of the systems components, which may imply why oscillations are not observed in all systems with negative feedback regulations. We went on to examine which distribution of the degradation rates would maximize oscillation occurrence, given they are required to maintain a constant steady-state level of the cascade output. We showed that for the single- as well as the dual-feedback motifs, symmetry among the elements within the feedback loops tends to promote oscillations, consistent with previous reports (Forger, 2010).

In this paper we employed deterministic modelling as the approach of choice and thus molecular fluctuations (noise) were neglected. However, these fluctuations are inherent features of biochemical networks and may be important in functioning of the cells (Balazsi et al., 2011). Negative feedbacks have been known to suppress noise (Hooshangi et al., 2006; Singh et al., 2009), understanding the behaviour of noise in nested feedback structures is an interesting topic. In our recent work (L. K. Nguyen et al., 2011), we explored how noise is propagated and managed in the prokaryotic tryptophan operon system of E.coli, a model system governed by three nested negative feedback loops. Interestingly, it was found that the individual loops in this system do not all reduce noise, but some actually enhance noise levels of the system output. This work was however only concerned with noise when the studied system is outside the oscillating domain. Therefore we further examined how noise might affect oscillations in the general dual-feedback motifs considered in the current work. Stochastic simulations showed that noise can extend the Hopf bifurcation point in both single- (F_{31}) and dual-feedback systems ($F_{31,32}$), producing oscillations at parameter values which does not lead to oscillations in a deterministic setting. Comparing the bifurcation points in F_{31} and $F_{31,32}$ at different levels of the inner loop showed that the points were not altered, suggesting that addition of the second loop in the nested arrangement had no effect on the stochastic bifurcation points. Further understanding concerning the interplay between noise and oscillations in coupled feedback settings should be among the focuses of future systems biology studies.

Recent progress in systems biology relies a great deal on numerical analysis and simulations. However, two important bottlenecks are identified with this approach: the lack of reliable parameter values and the limited exploring capability of numerical simulations. The former hurdle can be overcome by development of increasingly advanced experimental techniques; while one way to help reduce the second limitation is to develop novel analytical methods for quantitative analysis of cellular systems. Analytical tools can surmount brute-force simulations and boost the capacity to explore systems dynamics over much wider parameter space. The findings and implications in this study, as a result of such approach, have provided novel understanding of negative feedback regulation in coupling context. Our findings provide a novel understanding of negative feedback regulation in a complex cellular signalling context.

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FIGURE LEGENDS

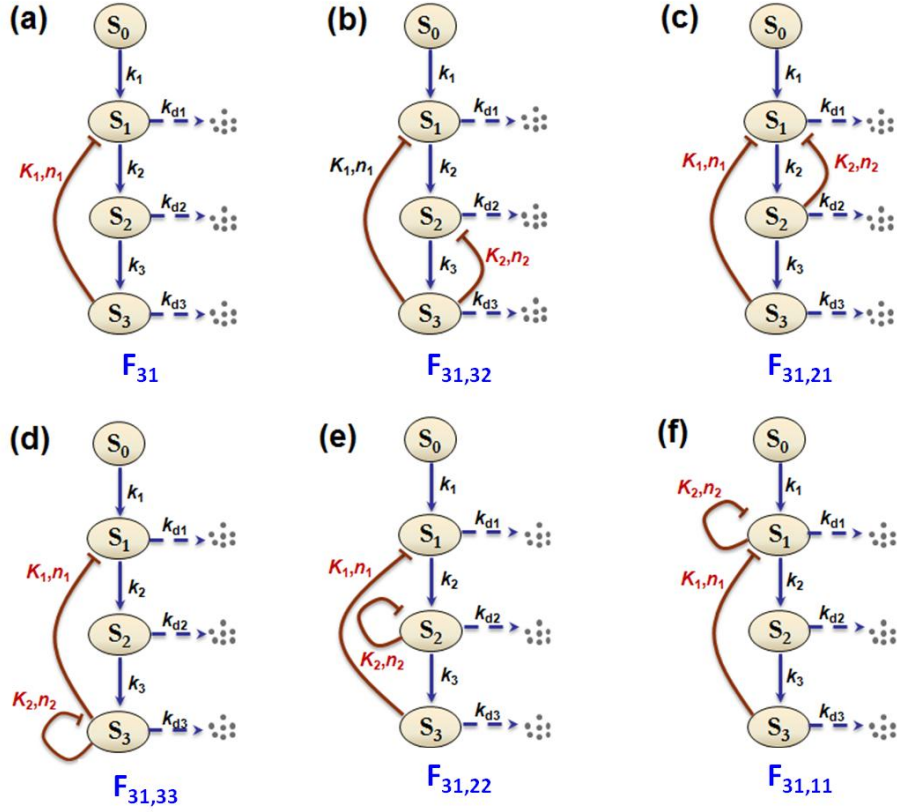


Figure 1. Kinetic schemes of the studied single- and dual-negative feedback motifs. Activation, inhibition and degradation are indicated by normal, barred and dashed arrows, respectively. The precursor S_0 is assumed to be constantly buffered. A single-loop motif where species S_i inhibits S_j is denoted F_{ij} ($i, j = 1..3$; $i = j$ indicates auto-regulatory loop where S_i represses itself). $F_{ij,kh}$ denotes a dual-feedback motif where S_i inhibits S_j and S_k inhibits S_h ($i, j, k, h = 1..3$). Following this notation, the feedback systems in this figure are referred to as F_{31} , $F_{31,32}$, $F_{31,21}$, $F_{31,33}$, $F_{31,22}$ and $F_{31,11}$.

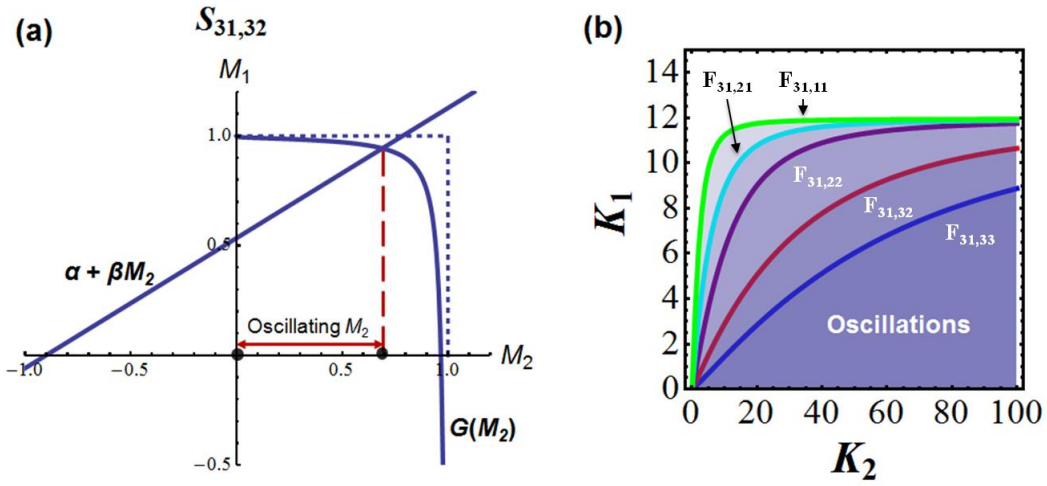


Figure 2. (a) Visualisation of the functions on both sides of eqn. (23), $\alpha + \beta M_2$ and $G(M_2)$, on the M_1 vs. M_2 coordinate which are treated here as dependent and independent variables. Range of M_2 indicated as ‘oscillating’ is obtained by determination of the functions’ intersection point within the 1×1 square. This range of M_2 gives rise to oscillations in system $F_{31,32}$ as explained in the text. Similar plots for the remaining dual-feedback motifs are presented in Fig.S3 ,ESM. Parameter values used for plotting are $K_1=1$ (nM), $K_2=2$ (nM), $k_1=1$ (nM min⁻¹), $k_2=k_3=1$ (min⁻¹), $k_{d1}=k_{d2}=k_{d3}=0.2$ (min⁻¹), $n_1=15$, $n_2=4$. (b) Bifurcation diagrams of fixed-point and oscillatory dynamics projected onto the K_1 vs. K_2 plane superimposed for all dual-feedback motifs. Parameter values used for plotting are $k_1=1$ (nM min⁻¹), $k_2=k_3=1$ (min⁻¹), $k_{d1}=0.15$ (min⁻¹), $k_{d2}=0.22$ (min⁻¹), $k_{d3}=0.18$ (min⁻¹), $n_1=9$, $n_2=2$. Note that the same parameter units are used for other figures hereafter.

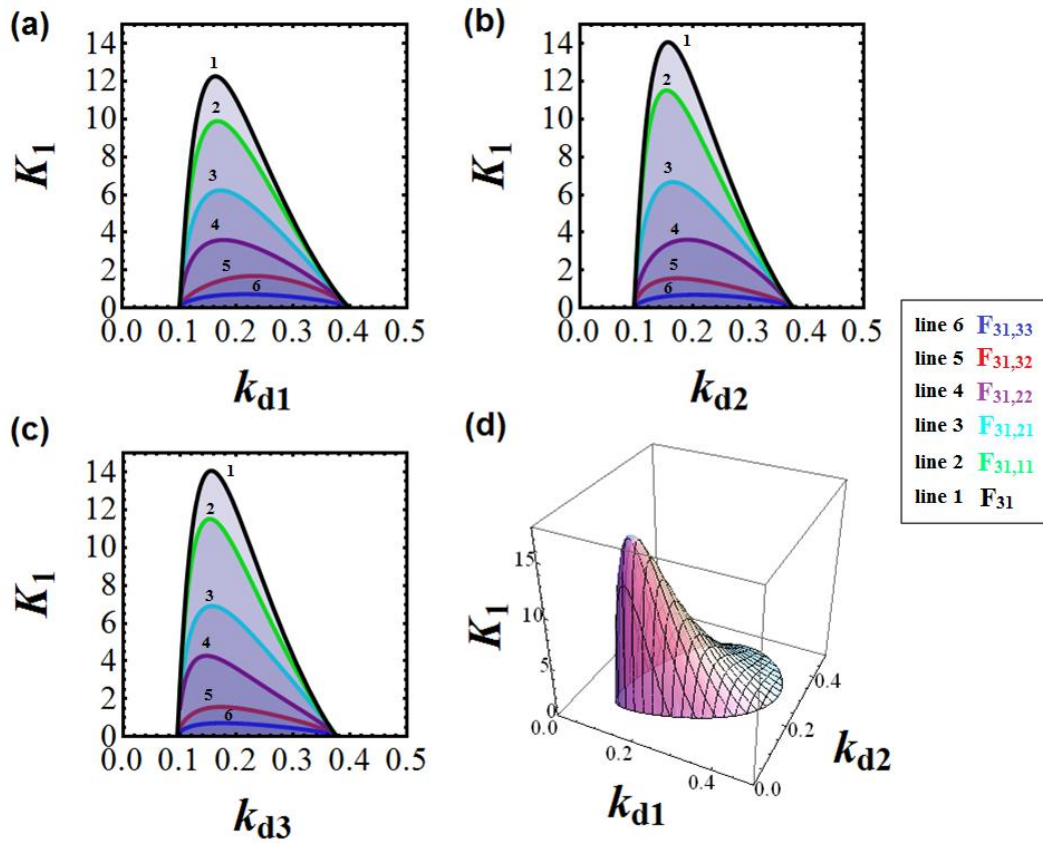


Figure 3. (a, b, c) Two-dimensional bifurcation diagrams of systems dynamics projected onto the plane of K_1 vs. rates of degradation superimposed for all dual-feedback motifs and compared to the single-feedback one. Oscillations domains are shaded. The results are consistent with observations on the K_1 vs. K_2 plane shown in Fig.2b. (d) Three-dimensional bifurcation plot which shows cone-shaped dependence of the threshold value of K_1 dependence on a pair of degradation rates, k_{d1} and k_{d2} , further illustrating the enclosing property of the oscillation domain in the degradation parameters' space. Parameter values used for plotting are $K_1=1$, $K_2=5$, $k_1=k_2=k_3=1$, $k_{d1}=k_{d2}=k_{d3}=0.2$, $n_1=9$, $n_2=2$. Note that the inner loops in all the dual-feedback motifs are specified with the same value of K_2 and n_2 for ease of comparison.

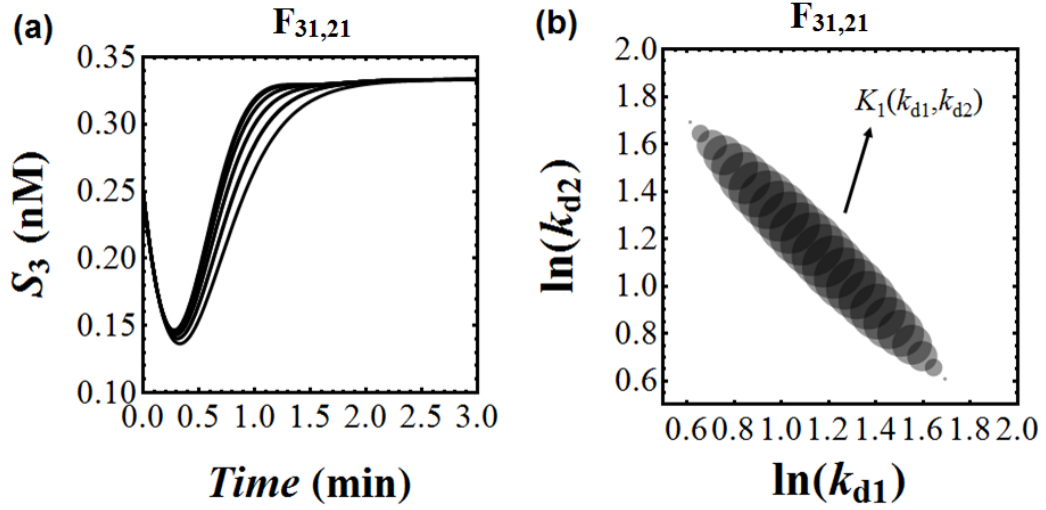


Figure 4. Dependence of the appearance of systems oscillations on the distribution of degradation rates. The dual-feedback $F_{31,21}$ is used here as a showcase, see Figure S4, ESM for the remaining motifs. **(a)** Time-course simulations showing that the constant steady-state level of S_3 is maintained by the condition of fixed $k_{d1} \cdot k_{d2}$, as discussed in the text. Each curve represents S_3 's temporal evolution simulated for one parameter set, with different $\{k_{d1}, k_{d2}\}$ but fixed $k_{d1} \cdot k_{d2} = 10$, and the remaining parameter values are $K_1=1$, $K_2=0.5$, $k_1=10$, $k_2=2$, $k_3=1$, $k_{d1}=2.5$, $k_{d2}=2$, $k_{d3}=3$, $n_1=10$ and $n_2=5$ (note that these values were chosen arbitrarily to illustrate our analytical results; a total of 200 parameter sets were generated). **(b)** For each parameter set discussed, we calculated the threshold value of K_1 which is shown as a dot whose size indicates its corresponding magnitude. Since $k_{d1} \cdot k_{d2}$ is fixed, all the dots line up on the log coordinate of k_{d1} and k_{d2} . The dots appear symmetrical and the largest dots are at the centre of the sets, showing that oscillations are most likely for sets with comparable k_{d1} and k_{d2} , and maximised with equal k_{d1} and k_{d2} .

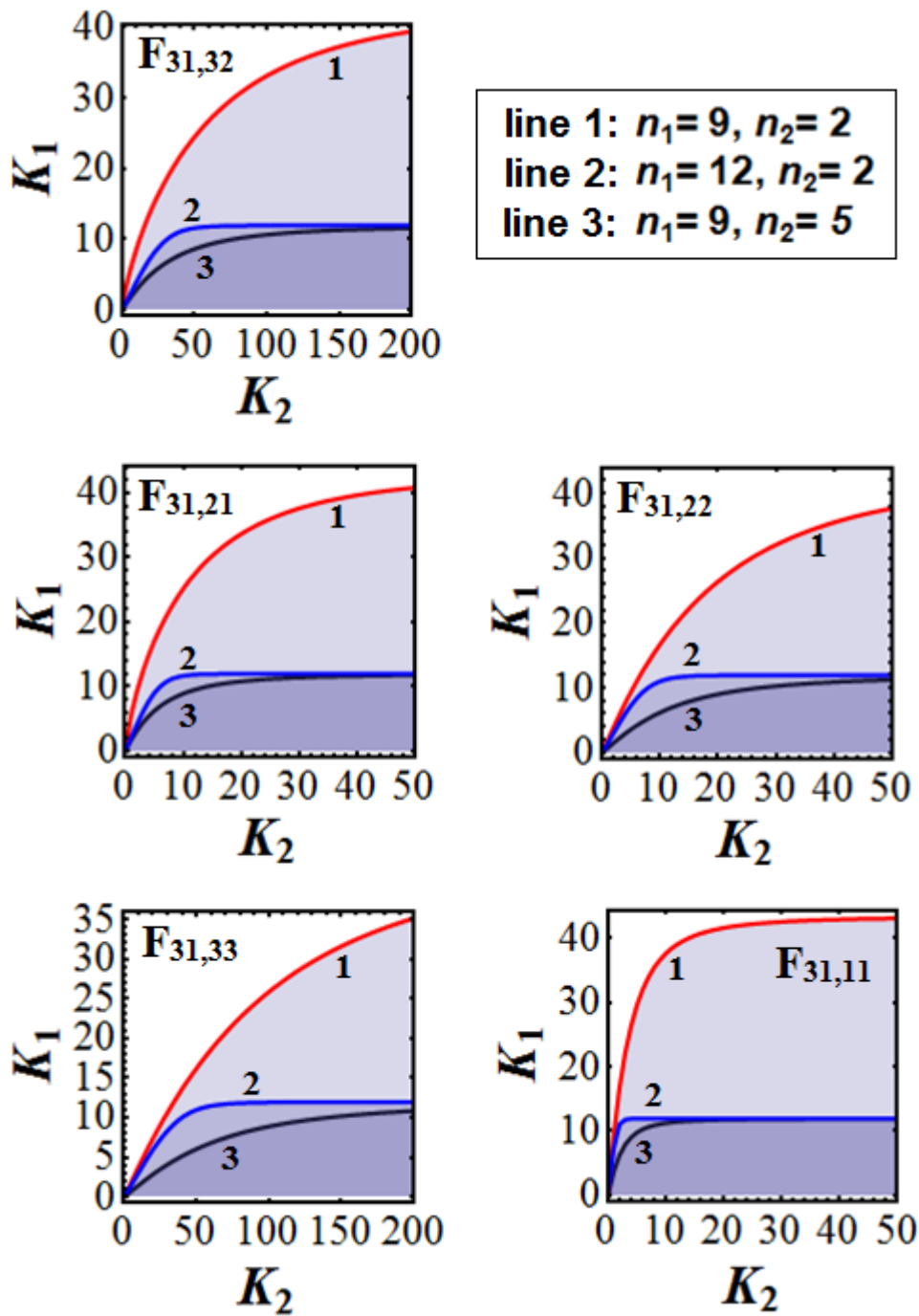


Figure 5. Effects of the feedback loops' nonlinearity levels on oscillations observed on the K_1 vs. K_2 bifurcation plot. For all the dual-feedback motifs, shaded oscillations domains are compared between a reference case (black) and when the first (red) or second loop (blue) is increased in Hill coefficient. The

remaining parameter values for plotting are $K_1=K_2=1$, $k_1=k_2=k_3=1$, $k_{d1}=k_{d2}=k_{d3}=0.2$.

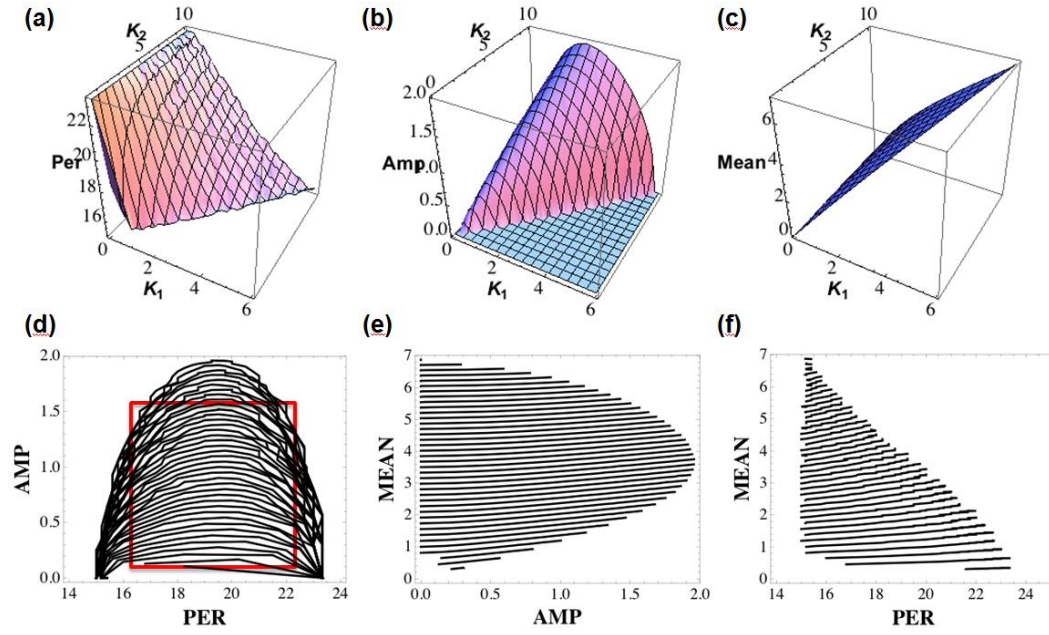


Figure 6. Regulation of the oscillations' period, amplitude and mean value in the dual-feedback motifs. **(a, b, c)** Dependence of the period (min), amplitude (nM) and mean value (nM) on simultaneous changes in the (reciprocal) strength of the outer (K_1) and inner (K_2) feedback loop. Parameters used are $k_1=k_2=k_3=1$, $k_{d1}=k_{d2}=k_{d3}=0.2$, $n_1=15$, $n_2=2$. **(d,e,f)** Same data obtained in (a,b,c) plotted in parametric plots for the period, amplitude and mean. **(d)** Each curve has different outer loop strength but identical inner loop strength; lower curves have stronger inner loop. **(e,f)** Each line has different inner loop strength but identical outer loop strength; the lower curves have stronger outer loop.

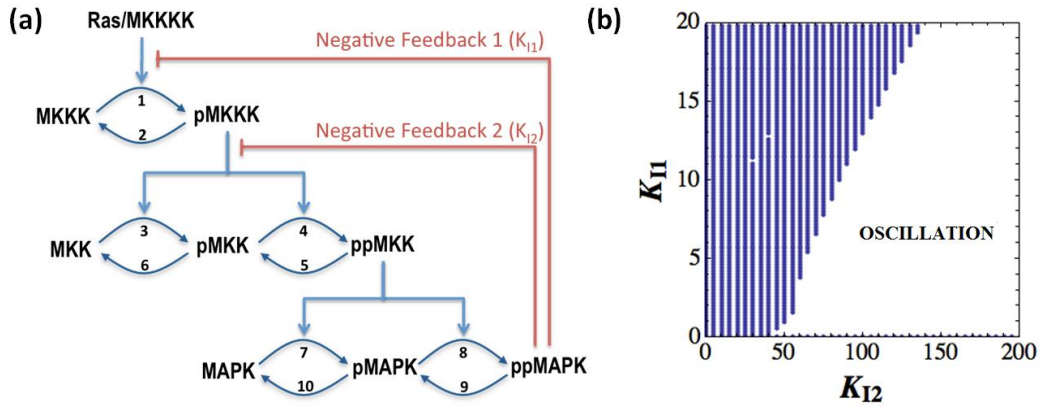


Figure 7. Analysis of oscillations in the dual-feedback MAPK cascades. **(a)** Kinetic scheme of the MAPK cascades extended from Kholodenko (2000); p- and pp- denote single and double phosphorylation of the MAPK protein, its kinase (MKK) and its kinase kinase (MKKK). Parameters K_{11} , K_{12} describe the reciprocal strength of the outer and inner feedback loop. **(b)** Bifurcation diagrams of fixed-point steady state (blue) and oscillatory dynamics (white) projected onto the K_{11} vs. K_{12} plane. Parameter values used are given in Table S1.

SHORT TITLE FOR PAGE HEADING

“Oscillations in dual-negative feedback motifs”.