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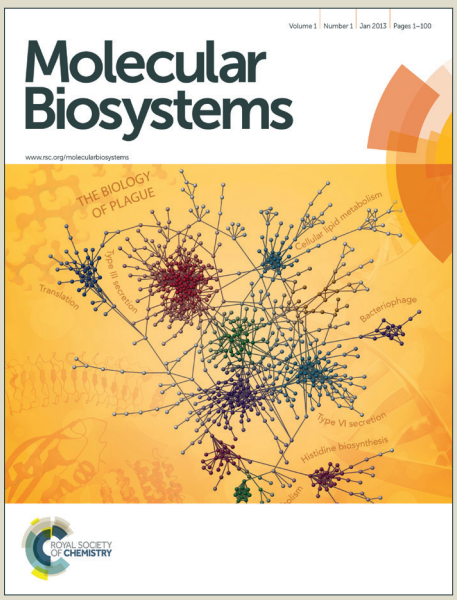

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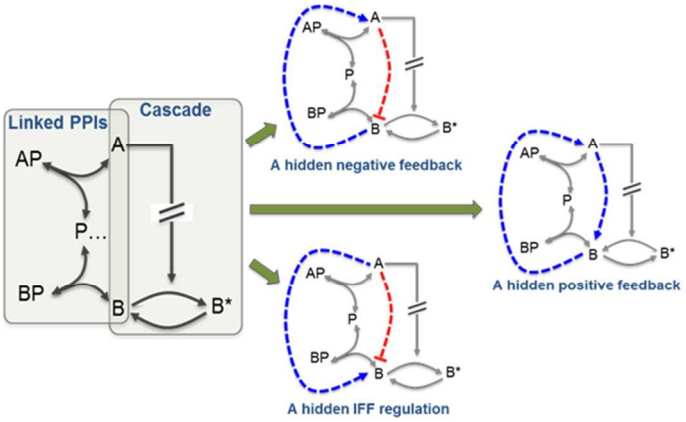
Novelty Summary and Graphical Abstract:

Our work reveals that simple reversible protein-protein interaction motifs, when being embedded into signalling cascades, could give rise to extremely rich and complex regulatory dynamics in the absence of explicit positive and negative feedback loops.



Protein-protein interactions generate hidden feedback and feed-forward loops to trigger bistable switches, oscillations and biphasic dose-responses.

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Abbreviations:

MST2: Mammalian STE20-like protein kinase 2
Hippo: Sterile 20-like kinase Hippo
Raf-1: v-raf-1 murine leukemia viral oncogene homolog 1
MEK: Mitogen-Activated Protein Kinase/ Extracellular signal-Regulated Kinase Kinase
ERK: Extracellular signal-Regulated Kinase
LATS1: Large Tumor Suppressor Kinase 1
RASSF1: Ras association (RalGDS/AF-6) domain family member 1
RKIP: Raf Kinase Inhibitor Protein

Abstract

Protein-protein interactions (PPIs) defined as reversible association of two proteins to form a complex, are undoubtedly among the most common interaction motifs featured in cells. Recent large-scale proteomic studies have revealed an enormously complex interactome of the cell, consisting of tens of thousands of PPIs with numerous signalling hubs. PPIs have functional roles in regulating a wide range of cellular processes including signal transduction and post-translational modifications, and de-regulation of PPIs is implicated in many diseases including cancers and neuro-degenerative disorders. Despite the ubiquitous appearance and physiological significance of PPIs, our understanding of the dynamic and functional consequences of these simple motifs remains incomplete, particularly when PPIs occur within large biochemical networks. We employ quantitative, dynamic modelling to computationally analyse salient dynamic features of the PPI motifs and PPI-containing signalling networks varying in topological architecture. Our analyses surprisingly reveal that simple reversible PPI motifs, when being embedded into signalling cascades, could give rise to extremely rich and complex regulatory dynamics in the absence of explicit positive and negative feedback loops. Our work represents a systematic investigation of the dynamic properties of PPIs in signalling networks, and the results shed light on how this simple event may potentiate diverse and intricate behaviours *in vivo*.

Introduction

Protein–protein interactions (PPIs) are fundamental molecular events and central to most biological processes, ranging from intra- and intercellular communication to cell-fate decision making [1-4]. Signal transduction, the propagation of signals from the cell surface to its interior, relies on a cascade of PPI events involving various interacting molecules. PPIs are also a hallmark of enzymatic reactions, where the reversible and transient binding of the enzyme and its substrate is a key initiating step. PPIs further underlie the formation of a wide variety of homo-oligomeric or hetero-oligomeric complexes. In addition, PPIs are commonly utilised by cells for regulatory purposes, to control protein activities and localization via PPI-mediated sequestration, or modulate pathway signalling using PPI-based scaffold proteins. Recent studies have further revealed interesting and essential roles of PPIs in cell-fates determination, by mediating the distribution of signals between different pathways [1, 2].

Recent advances in experimental techniques such as affinity purification coupled with mass spectrometry [5] and yeast two-hybrid screening [6] have enabled us to capture PPIs at the genome-wide level. These large-scale undertaking are revealing an enormously complex interactome of the cell, consisting of tens of thousands of PPIs with numerous signalling hubs [7]. Due to the widespread occurrence of PPIs in living cells, it comes as no surprise that aberrant PPIs are the underlying basis of many diseases, such as Creutzfeld-Jacob, Alzheimer's disease, and cancer [8, 9]. This is particularly notable in tumourigenesis, where the PPIs mediated signal transduction are often disrupted by mutations which result in distorted signal output and gene expression, leading to unrestricted proliferation of cancerous cells. Consequently, PPIs represent a large and important class of targets for human therapeutics [1, 10-12].

Yet, despite the ubiquitous appearance of PPIs, our understanding of the dynamic features and functional consequences of these simple motifs is poorly defined, due largely to the lack of systematic study of their regulation particularly when they form part of larger biochemical networks. A reason for this knowledge gap may be due to the seemingly simple nature of PPIs compared to more intricate and nonlinear signalling events such as phosphorylation or ubiquitination, which have received far more attention regarding their dynamic properties [13-16]. However, a closer examination shows that PPIs depend on multiple controlling parameters including binding affinities and concentrations of binding partners. PPI analysis is further complicated by the fact that many proteins have multiple binding partner resulting in competing PPI events [1]. Furthermore, the affinity of a PPI could be altered by post-translational modifications of the interacting protein partners, making PPI a potentially dynamic event [2]. Together, these parameters and their variable properties make it a non-trivial task to predict how the PPIs, in various wiring configurations, may impinge on systems dynamics.

Here, we employ quantitative dynamic modelling to computationally analyse the dynamic features of PPI motifs and PPI-containing signalling networks varying in wiring architecture. Our aim is to investigate the dynamic potentials of PPIs and distil principles governing their regulation. To this end, we study PPIs in isolation and as part of larger networks. Contrary to intuitive expectations, our analyses reveal that simple, reversible PPI motifs when being embedded into signalling cascades can bring about rich and complex regulatory effects characteristic of hidden negative, positive feedbacks and even feed-forward loop. These in turn give rise to highly nonlinear behaviours including bistable switches, oscillatory and biphasic responses. Using model-based dynamic analysis techniques, we explore the kinetic conditions that govern certain type of dynamic behaviours and how transition between different dynamic regimes could occur. We then discuss a number of physiologically relevant signalling systems in which PPIs play a central regulatory role and highlight salient dynamic features that these systems could exhibit. Our systematic investigation of the dynamic nature of PPIs in signalling networks shed light on how PPIs potentiate diverse and intricate behaviours *in vivo*. While explicit feedback and feed-forward loops are typically formed by post-translational modifications such as phosphorylation and

ubiquitination [2, 13], our results highlight that PPIs can result in hidden regulatory feedback and feed-forward loops.

Results

2. Single PPI motif linked to signalling cascades potentiates complex dynamic behaviours

2.1 Intrinsic bi-directional negative regulation in PPI motifs

To understand the kinetic behaviour arising from PPIs at the most fundamental level, we first examine the dynamic property of a single, isolated PPI motif. As illustrated in Fig.1, a simple reversible PPI event consists of two protein partners (A, B) associating to form a complex (AB) which can dissociate into the original proteins. The relative amount of the complex is determined by the affinity of the binding reaction. The kinetics of a PPI motif can be described by a set of ordinary differential equations (ODE) given in the Supplementary Information (SI), section S1.

Intuitive reasoning suggests that each of the PPI binding partners (A and B, Fig.1) exert an inhibitory regulation towards the other, through the formation of the AB complex. If the level of free A increases (decreases), the concentration of the AB complex increases (decreases), while the level of free B changes in the opposite direction to A, given the total affinity remains constant. The effect is schematically illustrated using the dot lines in Fig.1a,c. Likewise, raising or lowering the level of free B would result in lower or higher level of free A (Fig.1f, h). These relationships between A and B are illustrated by model simulations (Fig.1a-d, see also SI: S16). Perturbing A, thus, imposes a negative regulation towards B (Fig.1e). Due to the symmetrical property of the PPI motif, the converse is also true when protein B is perturbed (Fig.1f-i, and j).

Taken together, these analyses confirm existence of an intrinsic, bi-directional negative regulation between A and B in the isolated PPI motif, which we refer to as the PPI's terminal proteins (Fig.1k). This observation, although seemingly trivial at first glance, turned out to be dynamically significant and underlies a rich array of non-trivial dynamics when multiple PPIs are inter-linked and connected to molecular signalling cascades, as will be shown next.

PPI events rarely occur in isolation *in vivo*. Instead, they are often parts of larger molecular networks where the PPI's binding partners also take part in other biochemical reactions. Therefore, in order to understand the roles of PPIs in a more physiological context, we examine below the functional and dynamical features of the PPIs as they are connected to signalling cascades characterised by different wiring topologies.

2.2. PPI brings about and controls bistable switches

Signalling cascades are characterised by a series of chemical reactions that propagate cellular signals, typically from a cell-surface receptor to the cell interior. Signals are transferred from one cascade tier to the next, resulting in a signal output which often determines how the cell responds to the initiating cues. Signalling cascades are undoubtedly the most recognised structure that form the backbone of many signalling pathways [17, 18]. Consider a typical cascade where protein A acts as a regulator of B and facilitates the conversion of B into its modified form B*, which could be converted back to B by an opposing enzyme. *In vivo*, protein A could function as a kinase or an E3 ligase of B, while the opposing reaction may be catalysed by a phosphatase or a deubiquitinase. Fig.2a shows schematically a network scheme where the described cascade is linked to a single PPI event, where A and B constitute the binding partners. An ODE model of this network is given in the SI, S2. Of note, A may not necessarily be a direct enzyme catalyzing B-to-B* conversion but may act indirectly through a cascade of other enzymes.

Model simulations show that this linked network could give rise to bistable switches in the signal output (B* level) upon graded changes in inputs, under permissive parameter conditions. Bistability is demonstrated in Fig.2b that shows the steady-state dependence of B* level on the

increasing A abundance. Within a restricted domain of the A abundance, referred to as the bistable range, the steady-state level of B* could adopt either a high or a low value depending on the system's starting point. Bistability also brings about hysteresis that characterises an abrupt switch-off of B* as A becomes gradually more abundant, and an abrupt switch-on of B* at a different threshold as A abundance gradually decreases from a high value [17].

Although bistability most commonly results from a positive or double-negative feedback regulation [17, 19-21], in this case the PPI motif connecting A at the top of the cascade and B at the bottom of the cascade turned out to be responsible for the existence of bistability. In the absence of this PPI, a linear cascade from A to B is not capable of exhibiting bistability but can demonstrate only ultra-sensitivity [19, 22]. Moreover, we found that the affinity of the PPI, as given by the dissociation constant K_d, strongly controls the characteristics of bistability. A weaker binding between A and B by increasing the K_d could abolish bistability, while tighter binding renders more pronounced bistable behaviour (Fig.2b, inset). Thus, the presence of the A-B PPI potentiates and regulates bistability in this network.

To further understand how bistability arises, we used bifurcation analysis to analyse the dependence of bistability on model parameters. Besides requiring sufficiently strong PPI affinity, bistability was found to favour saturation of the modification reactions of B, i.e. low K_ms for either or both reactions (Fig.2c) which bring about the required nonlinearity. In seeking an intuitive explanation for bistability from a topological perspective, an underlying hidden double-negative feedback seems to emerge between A and B. We found that A exerts a negative regulation towards B by catalyzing its conversion to B*, while at the same time, B exerts a negative regulation towards A via the PPI motif (red dashed lines, Fig.2d), together constituting a two-way inhibition that gives rise to bistability.

2.3. PPI brings about oscillation and dynamics characteristic of feed-forward regulation

Next, we consider a network linking a PPI motif and a multi-tier cascade where A acts as a positive regulator of B via an intermediate protein C by catalyzing the conversion from B* to B (Fig.2e). Due to the intrinsic mutual negative regulation between A and B enabled by the PPI, we hypothesize that systems behaviours characteristic of both negative feedback and feed-forward regulation may arise under proper parameter regimes. To test this hypothesis, we first investigate if the network could display sustained oscillation, a hallmark feature of negative feedback [20]. Exploring the parameter space of an ODE model constructed for the network scheme in Fig.2e, we found the network could indeed exhibit sustained oscillatory behaviour, as depicted for B* in Fig.2f (model description is given in SI: S3). Sufficient time delay for the signal to propagate through the cascade was found to be required for sustained oscillation, as a shorter cascade where we removed the intermediate tier mediated by protein C, did not display sustained but only damped oscillation (SI: S4). The PPI's K_d strongly control the existence and shape of the oscillatory dynamics. Removal of the PPI completely abolished oscillation; while in its presence the oscillation amplitude displays a bell-shaped dependence on increasing K_d with larger amplitude within an optimal range of K_d value (Fig. 2f inset and Fig.2g). Together, these observations revealed PPI can brings about sustained oscillation, underlined by a hidden negative feedback effect between A and B, where A stimulates B via the cascade but is in turn suppressed by B via the PPI (Fig.2h).

We then investigate if the PPI could also induce effects of an incoherent feed-forward (IFF) loop regulation as A stimulates B but could concurrently suppress B via the PPI, due again to the bi-directional negative regulation (depicted in Fig.2i). IFF is among the most abundant network motifs found in biological systems [23], and a salient feature of its dynamics is characterised by a bi-phasic dependence of the level of the regulated on the level of the regulator [23, 24]. Our simulations showed that we could observe robust biphasic response of the steady-state level of B against a graded increase in A abundance, as shown in Fig.2j (ODE in SI: S5). Under these biphasic

response regimes, changing the A-B binding affinity have a strong effect on the biphasic response (Fig.2j, inset), influencing both the peak and optimal range of the response, which indicates a strong controlling role of the PPI motif for this behaviour. Removal of the PPI yields only a monotonic response instead.

Considering the scheme in Fig.2i again, it should now follow that the bi-directional negative regulation mediated by the PPI would also enable a coherent feed-forward (CFF) regulation between A and B. In this case, A suppresses B via two different routes: via the signalling cascade and via the PPI (Fig.2k, model description is given in SI: S6). Model simulations show a monotonic decreasing response of steady-state level of B against increasing A abundance (Fig.2n). A negative CFF regulation results in a strong inhibitory profile of B because of multiple negative regulations. Relieving one of the inhibitory routes would, in principle, reduce the degree of inhibition on B. To verify the CFF regulation in Fig.2n, we thus simulated the model by removing the PPI motif, which shows an up-regulation of the B dependence curve, suggesting that the complex formation event serves as an additional negative regulation of B by A. These observations in combination support the presence of a CFF effect from A to B under specific conditions.

2.4. Transition between distinct PPI-facilitated dynamics

By examining networks with similar topology but varying in number of cascade tiers and parameter values, our analyses have revealed that the inclusion of a single PPI motif in signalling cascades can induce a rich array of nonlinear, emergent systems-level dynamics including bistable switches, oscillations and biphasic responses. Interestingly, the inherent bi-directional negative regulation within a PPI enables these multiple dynamic features to co-exist under the same network structure. In one case, bistability could co-exists with CFF dynamics stemming from formation of positive feedback and CFF wirings; while in another case, oscillations may co-exists with bisphasic response as a result of negative feedback and IFF wirings. This property, where a single network structure can generate alternative wiring schemes and induce distinct dynamics is rather unique to networks containing PPIs, which have been so far largely overlooked.

Given the likely co-existence of different dynamics, we asked which factors may control the transition between one to another. Our model simulations suggest that depending on the PPI's binding affinity, either the negative regulation from A towards B or B towards A would become more prominent in the embedded network which favours formation of a specific regulatory mechanism, i.e. a negative feedback over an IFF, or a double-negative feedback over a CFF. Fig.3a-c show that as A-B binding affinity weakens (larger K_D), the system transitions from a sustained oscillatory regime to damped oscillations and then monostable with biphasic steady-state dose-response characteristics. Changing K_D thus has the ability to convert the system from being regulated by an implicit negative feedback to an implicit IFF. By the same token, increasing the PPI's K_D in the network schemes in Fig.3d,e could shift the system from being governed by a double-negative feedback regulation to a CFF regulation, subsequently replacing bistable response with a monotonic dose response. Together, these results indicate that the PPI not only cause the nonlinear systems-level behaviours, it plays determining role in mediating the transition between them.

3. Effects of coupled PPIs on network dynamics

3.1. Coupled PPIs possess intrinsic bi-directional positive regulation

Signalling proteins often have multiple binding partners. In many cases, protein associations occur in a mutually exclusive manner due to overlapping of the binding domains [2, 25, 26]. Motifs of coupled PPIs, where a protein P can bind partners A and B separately (presumably through distinct domains), are thus a common theme in biochemical networks. In a coupled PPIs motif, two single PPIs between A, B and the hub protein P are essentially linked together (Fig.4). Some

examples of coupled PPI motifs in physiological systems are given in SI: S7. We asked what effect the coupled PPI motifs may have on the systems dynamics when they are integrated in signalling cascades. We examined this question by first considering and modelling the coupled PPI motif in isolation, where P is simply shared by A and B (Fig. 4a). The model description, ODEs and parameter values are given in SI: S8.

Interestingly, our model-based simulations show existence of a bi-directional positive regulation between the terminal proteins A and B. Indeed, increasing (decreasing) the level of A results in a proportional increase (decrease) in the level of free B (Fig.4a-d). By symmetry, perturbing B also results in a corresponding change in the same direction in the level of free A (Fig.4f-i). The resulting mutual positive regulation between the terminal proteins of the coupled PPI motif (Fig.4e,j,k) is basically mediated through the component PPIs and could be intuitively explained as follows. As the level of A is increased, more P is recruited to form AP leaving less free P available for binding to B and so less B is required for this reaction, leaving more free B available. The opposite is true if A abundance is decreased instead. In effect, the bi-directional positive regulation between A and B is the net regulation of two sequential negative regulations between A and P, and P and B: A → B = A --| P --| B. As we will show below, this hidden two-way positive regulation confers the system to diverse non-linear systems-level dynamics.

3.2. Coupled PPIs render oscillation and biphasic responses of the same network

As in previous sections, we embedded the coupled PPI motif into classical signalling cascades as illustrated in Fig.5a (model description is given in SI: S9). Here, the cascade initiator, protein A, facilitates the conversion of B into its modified form B*, which can be reconverted into B by an opposing enzyme. Again, the cascade from A to B could span multiple tiers consisting of sequential interconvertible reactions. Exploration of model parameter space shows that the linked network can exhibit stable oscillatory dynamics, indicating the presence of an implicit negative and/or positive feedback mechanism (Fig.5b,c). An intuitive explanation for this negative feedback could be derived as follows: A negatively regulates B by converting it into B*, while B positively regulates A via the coupled PPI motif: A --| B → A (Fig.5b). We found that the relative binding affinities of the linked PPIs has a strong influence on the nature of oscillatory response. Denoting K_D(XY) as the dissociation constant of the X-Y binding, model simulations suggest that lower K_D(BP)/K_D(AP) induces more pronounced oscillations with larger amplitudes (Fig.5c, inset). It is interesting to compare the current scheme to that of Fig.2e. While both are capable of producing stable oscillations, the time delay needed for oscillation in Fig.2e stems from the multi-tier structure of the signalling cascade while it can arise from the coupling structure of the linked PPIs instead in the scheme Fig.5b, where the shared protein P serves to "delay" signal flow.

The same network wiring in Fig.5a can also exhibit an IFF's typical regulatory dynamics (SI: S10). Dose-response simulations of the network model show the characteristic biphasic character of the steady-state level of B on A abundance, indicating the presence of two concurrent but opposing regulations of B by A: A inhibits B via the cascade, and A stimulates B via the coupled PPI (Fig.5d,e). In this case, a higher relative binding affinity K_D(BP)/K_D(AP) appears to favour biphasic behaviour with higher peak and larger optimal range, indicative of more pronounced IFF regulatory effect in the network (Fig.5e, inset).

The intrinsic bi-directional positive regulation within a coupled PPIs motif thus renders the same network structure to potentially exhibit both oscillatory and biphasic dynamics. Moreover, the relative binding affinity K_D(BP)/K_D(AP) controls the transition between these behaviours (Fig.5f).

3.3. Coupled PPIs induce bistable switches

In contrast to the schemes in Fig.5a, we consider a variant network structure where A instead acts as a positive regulator of protein B by converting B*, a modified form of B, to B (Fig.5g). Model

simulations of this network (model description is given in SI: S11) show that the network is capable of generating robust bistable switching behaviour, as shown in Fig.5f. The steady-state level of B responds to increasing total level of A in a bistable fashion. In this case, the underlying positive feedback wiring responsible for bistability emerges from a positive regulation from A towards B via the cascade, and a positive regulation from B towards A via the coupled PPIs structure: A → B → A. Adding extra intermediate tiers to the cascade does not affect the existence of bistability. Analysis further shows that bistable dynamics are strongly determined by several model parameters. Particularly, we found that bistability is favoured at higher relative affinity $K_D(BP)/K_D(AP)$ (Fig.5h, inset). As the strength of the A-P binding affinity increases, the range of bistability becomes more pronounced.

4. Regulatory and dynamic consequences of multiply linked PPI motifs

The results above lead us to ask whether the observed bi-directional negative and positive regulations would still be an inherent feature of a linked PPIs motif with more than two PPI modules, and if so would intricate dynamics including bistability and oscillations still feature when these motifs are connected to signalling cascades. In short, we aim to generalise the result for motifs containing multiple PPIs linked together.

Indeed, intuitive reasoning consolidated by model simulations for a motif linking three PPI modules, illustrated in Fig.6c, showed that perturbing one of the terminal proteins (A or B) up or down would result in lower or higher level of the other, respectively. This suggests that, similar to the case of a single PPI, a 3-PPI motif possesses an intrinsic bi-directional negative regulation due to the structural symmetry. On the other hand, a similar analysis for a 4-PPI motif revealed a bi-directional positive regulation between the terminal proteins (Fig.6d), as in the case of the coupled PPI motif. These observations in combination, allow us to draw a generalised conclusion, that is bi-directional regulation is an intrinsic feature of linked PPIs motifs where the terminal proteins exert a negative effect towards each other if there are an odd number of the PPI modules, but a positive effect if there are an even number of the linked PPIs (Fig.6c,d). Not only that, we found that the dynamic consequences of these motifs are also preserved when they are integrated into signalling cascades. Depending on the direction of signal flow within the signaling cascade (A as a positive or negative regulator of B), a linked PPIs motif with an odd or even number of modules would still bring about sustained oscillations, bistable and biphasic responses.

5. Dynamic consequences of PPIs in in vivo biochemical systems

Owing to their fundamental nature, PPI motifs appear ubiquitously in biochemical networks. At the molecular level, PPIs are mediated by various protein binding domains. SI: S17 surveys common protein domains that facilitate its binding to other proteins or lipids, showing that PPIs occur in a multitude of signalling systems. Some protein domains also interact with more than one domains, giving rise to linked PPI mechanisms within signalling systems. Taken together, the widespread occurrence of PPIs *in vivo* suggests potential for rich and diverse systems-level behaviours for biological systems containing these motifs. In this section, we examine two examples of physiological systems in which PPIs are present, and study their dynamic potentials.

5.1. The MST2/Raf-1 signalling network:

Originally identified in the fruit fly *Drosophila melanogaster* through genetic screenings for growth suppressors, the MST2/Hippo signaling pathway has emerged as a an important pathway for the regulation of growth, apoptosis and proliferation in mammalian cells [1, 27]. Recently, using a combined experimental-computational systems approach we have elucidated the dynamic crosstalk between the pro-apoptotic MST2 and the pro-proliferative Raf-1 pathway occurring at several levels [1, 2]. These crosstalks coordinate to orchestrate the opposing activities of the pathways. At the heart of the crosstalk are dynamic changes in PPIs between the kinases MST2 and Raf-1 and their respective upstream activators RASSF1A and Ras. In particular, the PPI

affinity between MST2 and Raf-1 is strongly mediated by the phosphorylation status of the partner proteins, where inactive phosphorylated MST2 binds much more tightly to inactive Raf-1. We also worked out a cross-pathway feedback that connects MST2 signalling to Raf-1 activation where LATS1, a cognate substrate of MST2, inactively phosphylates Raf-1 and promotes its binding to MST2 [1, 2].

A simplified scheme of the core MST2/Raf-1 interaction circuitry is given in Fig.7a. Interestingly, slightly rearranging this circuitry transforms it into a familiar wiring seen in Fig.2e which integrates the (inactive) pMST2/pRaf-1 complex (PPI) with the MST2 → LATS1 --| Raf-1 signalling cascade. Expectedly, a model constructed for the scheme predicts that the MST2/Raf-1 system could generate sustained oscillation and biphasic response (Fig.6b,c; model description given in SI: S12). Oscillation has not been documented so far for this system. However, our analyses of the dynamics governed by PPIs, when applied to the context of the MST2/Raf-1 crosstalk, have unveiled a rather intriguing possibility of sustained oscillatory dynamics in this system. Related questions such as whether such predicted oscillation and biphasic response do really manifest in certain physiological contexts, and if so, what the likely roles of oscillations would be interesting future avenues for investigation.

5.2. The Raf/RKIP/MEK signalling network

The MAPK pathway is a classic signalling pathway known to be involved in regulating cell growth, proliferation and apoptosis [13, 28]. The pathway is dysregulated in many diseases, including cancer, making this pathway the focus of many therapeutic studies [29, 30]. The signal flows as phosphorylation events propagate from Raf to ERK via MEK: phosphorylated and active Raf triggers MEK activation which in turn phosphorylates and activates ERK. Active ERK could inhibit Raf through an inhibitory phosphorylation, resulting in a negative feedback regulation [31, 32]. The importance of protein interactions regulating the signal flux through this pathway has only recently been recognised. We identified RKIP is an endogenous inhibitor of MAPK signalling [33]. Mechanistically, RKIP binds and sequesters Raf-1 from activating MEK. Interestingly, RKIP was also found to bind to MEK, and furthermore Raf-1 binding to RKIP and that of MEK are mutually exclusive [33, 34]. RKIP is thus a physiological endogenous inhibitor of both Raf-1 and MEK. A simplified schematic diagram of this signalling network presented in Fig.7d shows that network contains two PPIs formed between Raf-1, MEK and the shared inhibitor RKIP, which are coupled and linked to the Raf/MEK/ERK cascade (model description in SI: S13). Although the dynamic behaviour of the Raf/MEK/ERK cascade focusing on the role of RKIP was investigated to some extent [35], the RKIP-mediated coupled PPIs motif was not under investigation. Here, we are specifically interested in the potential systems dynamic features that may be attributed by the presence of the RKIP-mediated PPIs.

From our previous results, graphical study of the network structure suggests the existence of three possible oscillations generating mechanisms. First, the negative feedback from ERK to Raf-1 has been known to bring about oscillations (Fig.7e) [36]. Secondly, due to the coupled PPIs motif mediated by RKIP, a hidden negative feedback exists between Raf-1 and MEK where the inactive MEK positively regulates Raf-1 via the PPIs while being suppressed by Raf-1 via the cascade (Fig.7g). Finally, assuming MEK activation may be additionally induced by Raf independent kinases, there exists another hidden negative feedback from MEK to Raf-1 where Raf-1 stimulates inactive MEK via the PPIs, while being suppressed by MEK through ERK-mediated inhibitory phosphorylation (Fig.7i). Reassuringly, model simulations of the network where we alternatively retained only the reactions required for each mechanism (while non-required reactions are grayed out in Fig.7e,g and i) showed sustained oscillation under proper parameter conditions (Fig.7f, h and j), confirming that all three mechanisms alone could generate this dynamics.

Our results have thus suggest novel mechanisms driven by PPIs that the Raf-1/MEK/ERK pathway may utilise to produce oscillatory dynamics. It is possible that the three discussed mechanisms may all contribute to oscillations under conditions observed *in vivo*.It is reasonable also to expect that some of these mechanisms may be more prominent in distinct cellular settings due to variations in protein expression and/or network wiring between cell and tissue types. Nevertheless, a more detailed integrated study examining the possible interplay of these mechanisms in specific cellular platform and the dynamic consequences would further shed light on the functional role of RKIP as a signalling modulator. This task becomes even more urgent given the increasing importance of RKIP as a suppressor of metastasis in cancers and a potential therapeutic target [37, 38].

5.3. The PDK1/14-3-3/YAP1 signalling network

The Akt pathway is an important and well-studied signalling system because of its central role in controlling cell proliferation, apoptosis and tumourigenesis [39]. Many cancers show an altered activity of this pathway making it the target of promising therapeutic strategy. PI3K generates PIP_3 and recruits PDK1, which phosphorylates and activates Akt that in turn phosphorylates the transcriptional regulator, YAP1. Akt-mediated phosphorylation of YAP1 facilitates its binding to an adapter protein, 14-3-3, thereby sequestering YAP1 to the cytoplasm [40]. Interestingly, 14-3-3 has also been reported to bind and sequester PDK1 [41]. Therefore, 14-3-3 sequesters multiple proteins generating a coupled PPIs motif. The schematic representation of the signalling reactions (shown in Fig.7k), includes a cascade of phosphorylation events from PDK1 to YAP1 via Akt and a coupled PPIs of 14-3-3 with PDK1 and pYAP1.

We constructed an ODE model for the PDK1/14-3-3/YAP1 network (see SI: S14), and our model simulations showed that under certain parameter conditions, the network could exhibit bistable behaviour (Fig.7l). As an example, dose-response simulations of pYAP1 against increasing total PDK1 display bistable switch behaviour for pYAP1: for a certain range of total PDK1, the pYAP1 level may be either high or low depending upon their previous state. As observed previously, the binding affinities of 14-3-3 to PDK1 and pYAP1 have an important role in governing the characteristics of bistability. Stronger relative binding affinity of 14-3-3 with PDK1 (high K_d(14-3-3.pYAP1)/K_d(14-3-3.PDK1)) enhances bistability as seen in Fig.7m depicting the 2D bifurcation diagram between the binding ratio and 14-3-3 abundance. In this circuit, bistability arises from a hidden positive feedback regulation in the system: PDK1 positively regulates the phosphorylated inactive YAP1 via Akt, while phosphorylated YAP1 positively regulates PDK1 via the coupled PPI wiring with 14-3-3. Thus, our simulations predict that bistability can arise in the Akt signalling network through a novel mechanism: protein sequestration by 14-3-3.

Discussion

Aberrant signalling drives the pathological behaviour of cells during disease development, such as in tumourigenesis. As cancer is ultimately a phenotypic manifestation of malfunctions often arising at the signalling network levels, to understand how inappropriate cellular decisions are made and how to optimally modulate them, we need to gain in-depth understanding of the dynamic properties of these networks. Such understanding holds great promise for the development of cancer therapeutics. However, due to the complexity and nonlinearity of signalling networks that involve a large ensembles of dynamic interactions in space and time [17], our knowledge of the dynamic properties of signalling networks are far from complete. Valuable tools including computational modelling and quantitative biology are taking centre stage to elucidate network dynamics and identify novel therapeutic strategies [1, 13].

In the present study, we exploited kinetic modelling and model-based analysis to systematically investigate the salient dynamic features brought about PPIs, arguably the most abundant and fundamental motif in biochemical networks. Our analyses show that an array of highly complex

systems-level behaviors can be brought about by the intrinsic properties of PPIs that are embedded in signalling networks. Most notably, the intrinsic nature of (positive and negative) bi-directional regulations between the terminal proteins of single or linked PPI motifs have led to existence of bistable switches, sustained and damped oscillations, and biphasic steady-state responses in various signalling networks, intriguingly in the absence of any external regulatory loops. Furthermore, due to this bi-directional effect, PPI-containing networks could potentially exhibit co-existence of dynamics characteristic of either positive (negative) feedback or feed-forward regulation using the same network design. It should be noted that for simplicity we described the enzyme-catalysed reactions in our models using Mechaelis-Menten kinetic law. However, the results are still consistent when we modelled using mass-action kinetics where all the enzyme-substrate complexes are explicitly described. Nevertheless, to avoid potential duplication of complex formations by enzyme-catalysed reactions and by PPIs, in certain cases more than one intermediate tier are required in the cascade for observation of oscillations.

Bistable and ultrasensitive switches are among the most common dynamics exploited by cellular machineries to convert analog signals into digital outputs, which enable cells to make clear-cut and unambigious cell-fate decisions [19]. So far, a multitude of underlying molecular mechanisms capable of generating these switches have been identified (reviewed in e.g. [42]). Explicit positive and double-negative feedback regulations are the earliest and major mechanisms known to drive bistability. We have later identified more subtle mechanisms that do not involve any explicit feedbacks yet able to produce bistability, including multistep phoshorylation [43] and polyubiquitin chain formation [16]. The current work further adds to this repertoire of non-trivial mechanisms by revealing simple protein-protein interactions, when properly connected to signalling cascades, could also trigger robust bistability.

We extended our general analysis to three physiological signalling systems, the Raf-1/MEK/RKIP, MST2-Raf-1 and PDK1/14-3-3/YAP1 networks which feature different PPI-related designs. In the first system, RKIP serves as a shared endogenous inhibitor of both Raf-1 and MEK and gives rise to a coupled PPIs motif. We showed that this motif confers additional routes through which the ERK MAPK network could generate oscillations independently of the classic ERK-to-Raf negative feedback. The result suggests new possibility that oscillatory dynamics observed experimentally could be partly attributed to the PPI-related mechanism. From a dynamic perspective, it would be interesting to study how these distinct oscillation-generating mechanisms interplay, and whether they synergize/antagonize in producing oscillations. In the second examined system, crosstalk between MST2 and Raf-1 features a single PPI motif between inactive forms of these proteins. In this case, post-translational modifications of MST2 and Raf-1 strongly affect the PPI. This single PPI when connected to the MST2-LATS1-Raf-1 signalling cascade constitutes a network design that was found to give rise to sustained oscillation and biphasic response. We have previously analyzed the circuitry of the MST2-Raf-1 crosstalk and shown the robust presence of signalling switches [1, 2]. The current work further unveils an intriguing possibility for novel complex dynamics that this integrated network may feature in physiological contexts. Such predictions will be interesting to verify experimentally. In the last system, our simulations predict that bistability can arise in the Akt signalling network through a novel, hidden positive feedback mechanism mediated via PPIs involving 14-3-3. Taken together, our analyses of realistic signalling systems highlight that bistable switches, oscillation or bisphasic responses could arise in physiological signalling networks without the explicit presence of positive, negative feedback or feed-forward loops, but rather as a consequence of interconnected protein-protein interactions. Not only our results have unveiled a capacity for displaying surprisingly rich dynamics by PPI-containing networks, the ubiquitous appearance of PPIs implies that nonlinear dynamics such as bistability, hysteresis switches, oscillation and biphasic response may be more widespread than previously expected.

The relevance of PPIs and their consequence in systems dynamics is expected to become even more significant as more intricate PPI-related motifs are unveiled. Our work currently considers a particular coupling strategy where PPIs are sequentially linked. However, experimental evidence suggests a large library of different design principles where multiple PPIs could be linked (e.g. see SI: S15). For example, a signalling hub protein being shared by multiple binding partners in a mutually exclusive manner is a common theme in cells. Analysis of the ErbB signalling network, for example, has revealed a large number of such competitive, mutually exclusive protein interactions [25]. Understanding the dynamic capability of these PPI networks at the systems level through systematic analysis of the component PPIs and their integrated, emergent behaviour will be important to elucidate the functional roles of these networks in pathophysiological contexts. The results in this work will provide a foundation for such investigation.

As PPIs are emerging as an important mechanism to regulate and distribute signals between diseases related pathways, targeting PPIs for therapeutic purpose is receiving increasing attention by the scientific community [1]. The relevance of PPI as putative therapeutic targets for the development of new treatments is particularly evident in cancer, with several on-going clinical trials within this area [44, 45]. In this context, a major implication of the present work involves the potential consequences on systems dynamics that inhibitor agents may induce. This is because binding events play a major role in determining the dynamics and kinetics of drug agent activity. Apart from intended strong binding to the target protein, drug agents often have weaker (and likely reversible) off-target bindings which could constitute coupled PPI and multi-liked motifs. Insights into the dynamic features of these PPI motifs in drug related contexts may be relevant to understand drug-target response and limit drug side effects.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Information

Description of all mathematical models including reaction rates, ODEs, and parameter values used for plotting are described in details in the Supplementary Information.

Figure legends

Figure 1. A single PPI motif possesses intrinsic bi-directional negative regulation. A PPI event was modelled and simulated under different perturbation scenarios. The system was allowed to reach steady state (1 hr) and then the respective perturbations were introduced (from 1 hr to 2 hr). Protein A's abundance is increased **(a,b)** or decreased **(c,d). (e)** A negatively regulates B. Similarly, protein B's abundance is increased **(f,g)** or decreased **(h,i). (j)** B negatively regulates A. (**k)** Taken together, there exists a mutual negative regulation between A and B in a single PPI motif. Parameters used for simulations are given in the SI.

Figure 2. Single PPI motif linked with signalling cascades display highly non-linear systems behaviours. (a) Schematic diagram of an integrated network linking a single PPI motif to a classical signalling cascade where A acts as a catalysing enzyme for B. **(b)** Model simulations show bistable switches for B* concentration against increasing A abundance. Inset figure compares the hysteresis curves for different dissociation constant values for A-B binding. **(c)** A 2D bifurcation plot showing dependence of bistability on changes in the Michealis-Menten constant Kms of the interconversion reactions for B. **(d)** Graphical illustration of a hidden double-negative feedback emerging from the nework structure. **(e)** Schematic diagram of an integrated network linking a single PPI motif to a multi-tier cascade where A positively regulates B* to B conversion. **(f)** Temporal sustained oscillation simulated for B* concentration. **(f)** Dependence of the oscillation amplitude on change in A-B's dissociation constant. **(h)** Illustration of a hidden negative feedback emerging from the nework structure. **(i)** A hidden incoherent feed-forward regulation emerging from the nework structure that leads to bi-phasic dose-response. The double slash symbol indicates the regulation by A could be indirect **(j)** of B concentration against A abundance. **(k)** A hidden coherent feed-forward regulation emerging from the nework structure that leads to monotonic dose-response **(h)** of B concentration against A abundance. Parameters used for simulations are given in the SI.

Figure 3. Transitions between the hidden regulatory mechanisms and consequent systems-level dynamics in the integrated networks considered in Fig.2. (a-c). Change in the dissociation constant governing the A-B PPI could shift the same system from a negative feedback to a IFF regulatory mode. (d-f) On the other hand, change in this dissociation constant could shift the same system from a double-negative feedback to a CFF regulatory mode.

Figure 4. Coupled PPIs motif possesses intrisic bi-directional positive regulation. A coupled motif connecting two PPIs was modelled and simulated under different perturbation scenarios in a similar manner as described in Fig.1. Parameters used for simulations are given in the SI.

Figure 5. Non-linear systems behaviours arising from coupled PPIs motif. (a) A coupled PPIs motif when integrated into a cascade where A converts B to B* could geranate two co-existing regulatory modes: a hidden negative feedback regulation **(b)** leading to oscillation **(c)**; or a hidden incoherent feed-forward regulation **(d)** that leads to bi-phasic dose response dependence. **(f)** Transition between the above regulatory modes is controlled by the relative dissociation constants between B-P and A-P bindings. **(g)** An alternative network structure that links a coupled PPI motif to a signalling cascade where A converts B* to B, is predicted to display bistable switches **(h)**. Parameters used for simulations are given in the SI.

Figure 6. Generalization of the intrinsic, mutual regulation within linked PPI motifs. The symmetric nature of the sequentially linked PPIs motifs facilitates mutual regulation from A to B and B to A, mediated via the intermediate binder proteins P_is. For motifs containing an odd number of PPIs such as in **(a)** and **(c)**, a mutual negative regulation exists between the terminal proteins A and B; while a a mutual positive regulation features when there is an even number of PPIs, as in **(b)** and **(d)**.

Figure 7. Model-based analysis of systems-level dynamics mediated by PPIs in the MTS2/Raf-1, Raf/MEK/RKIP and PDK1/14-3-3/YAP1 networks. (a) A schematic diagram of the simplified MST2/Raf-1 network featuring a single PPI motif. **(b,c)** Co-existence of negative feedback and IFF regulatory mechanism leading to either oscillation or bi-phasic dose-response. **(d)** A schematic diagram of the simplified Raf/MEK/RKIP network featuring a coupled PPI motif mediated by RKIP. **(e,g,i)** Illustration of three different negative feedback mechanisms existing in the network each alone could generate sustained oscillations, observed in **(f,h,j)** respectively. **(k)** A schematic diagram of the simplified PDK1/14-3-3/YAP1 network featuring a coupled PPI motif mediated by 14-3-3. **(l)** Bistable switch predicted for pYAP1 against increasing PDK1 abundance. **(m)** A 2D bifurcation diagram showing the dependence of existence for bistability (purple region) on changes of the 14-3-3 abundance and the binding affinity ratio between the component PPIs. All models' description and parameter values used for simulations are given in the SI.

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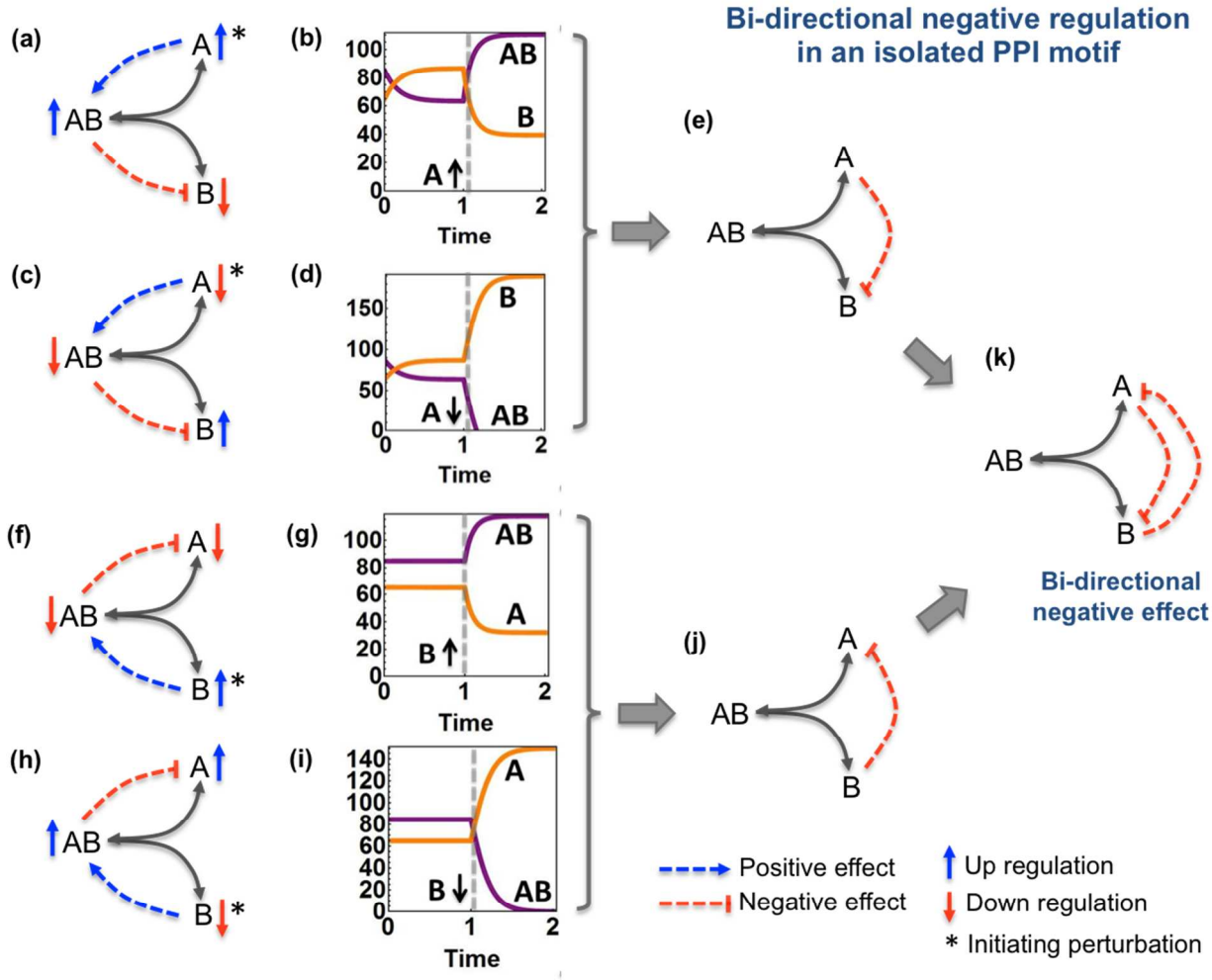

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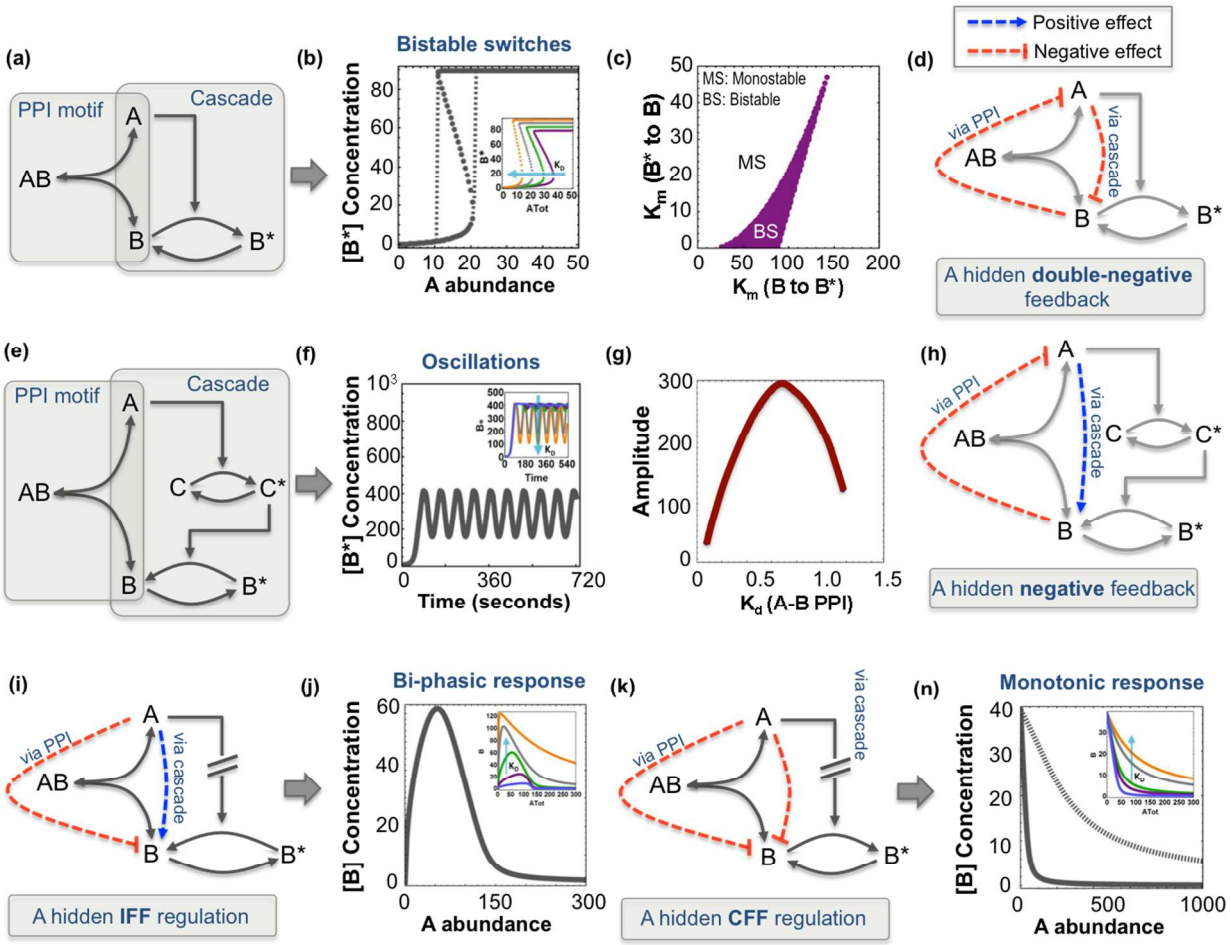

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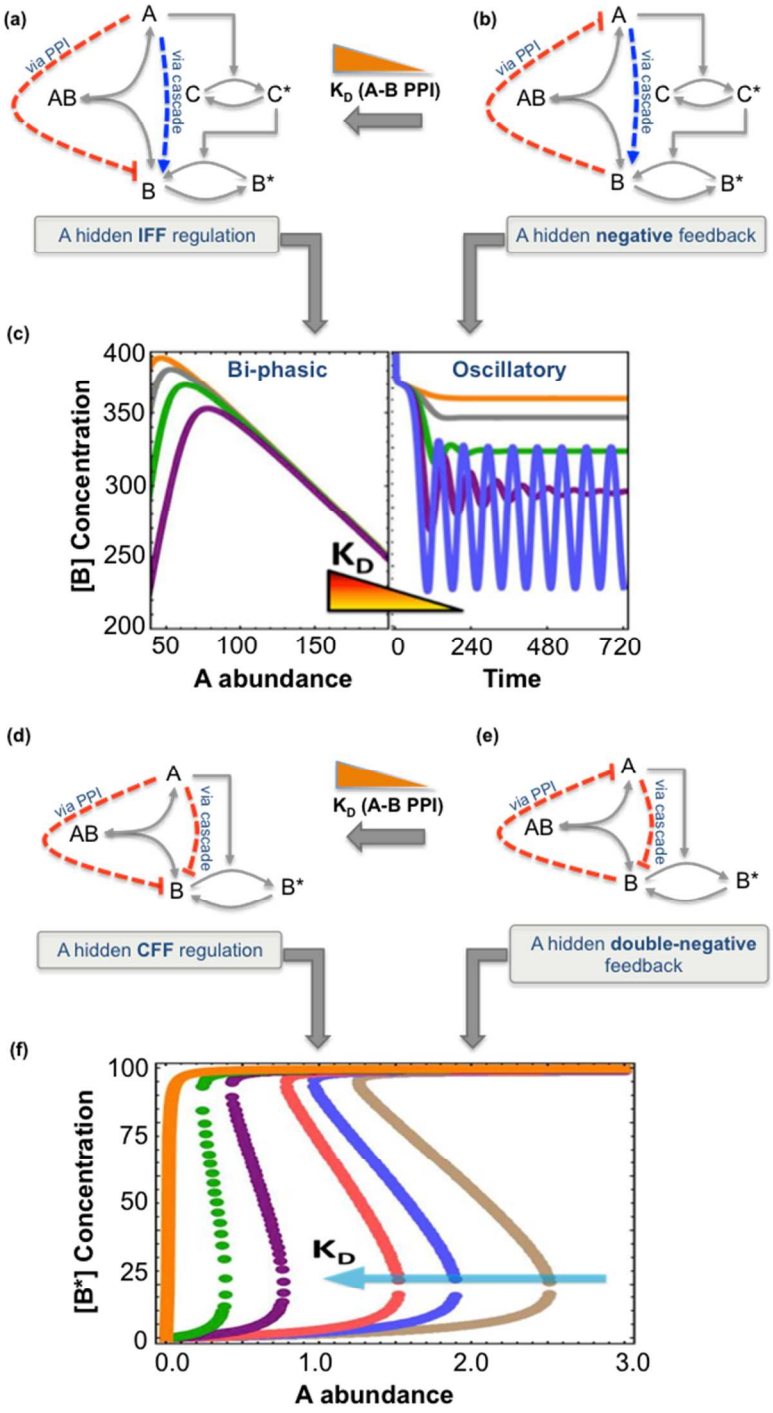

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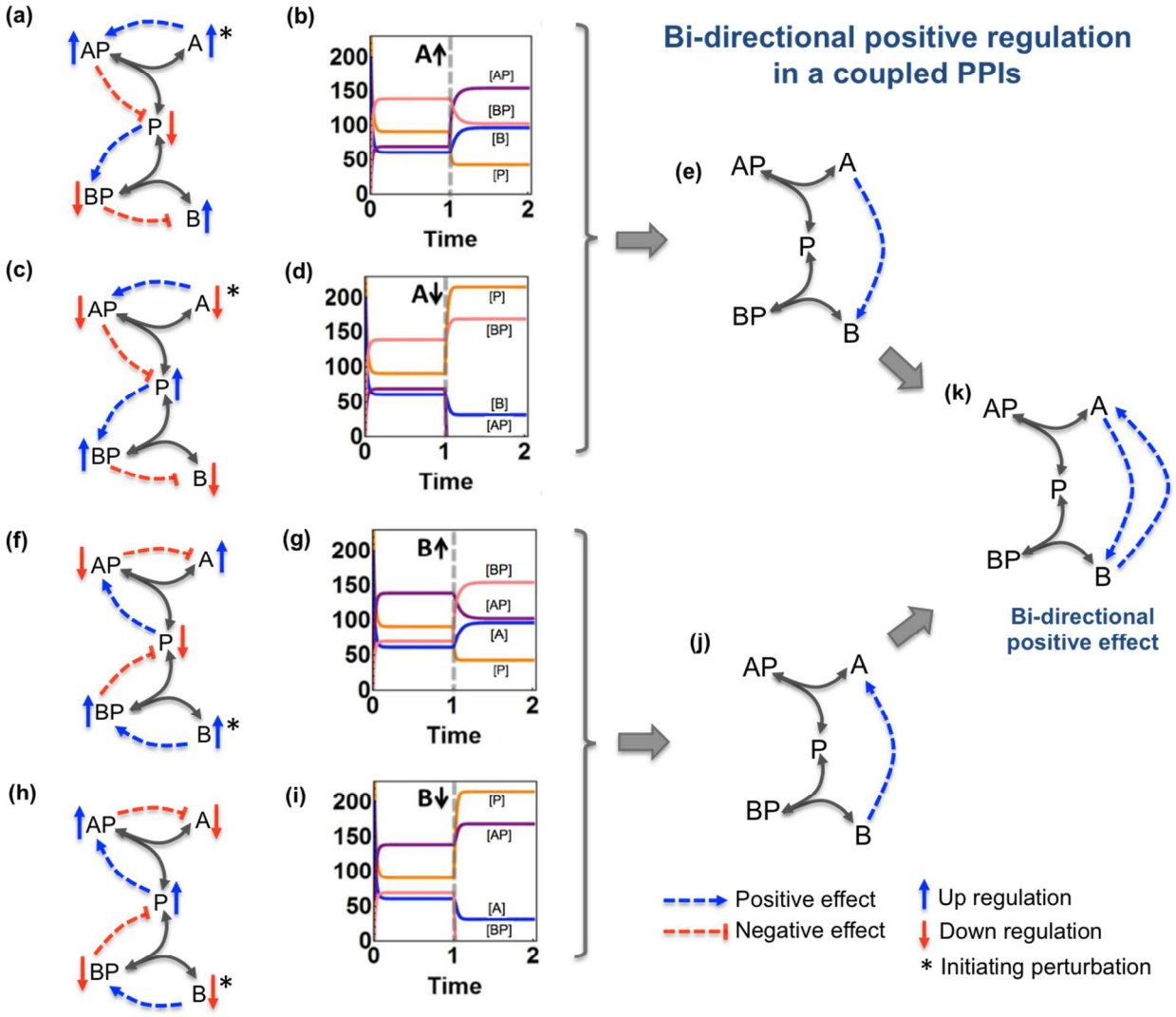

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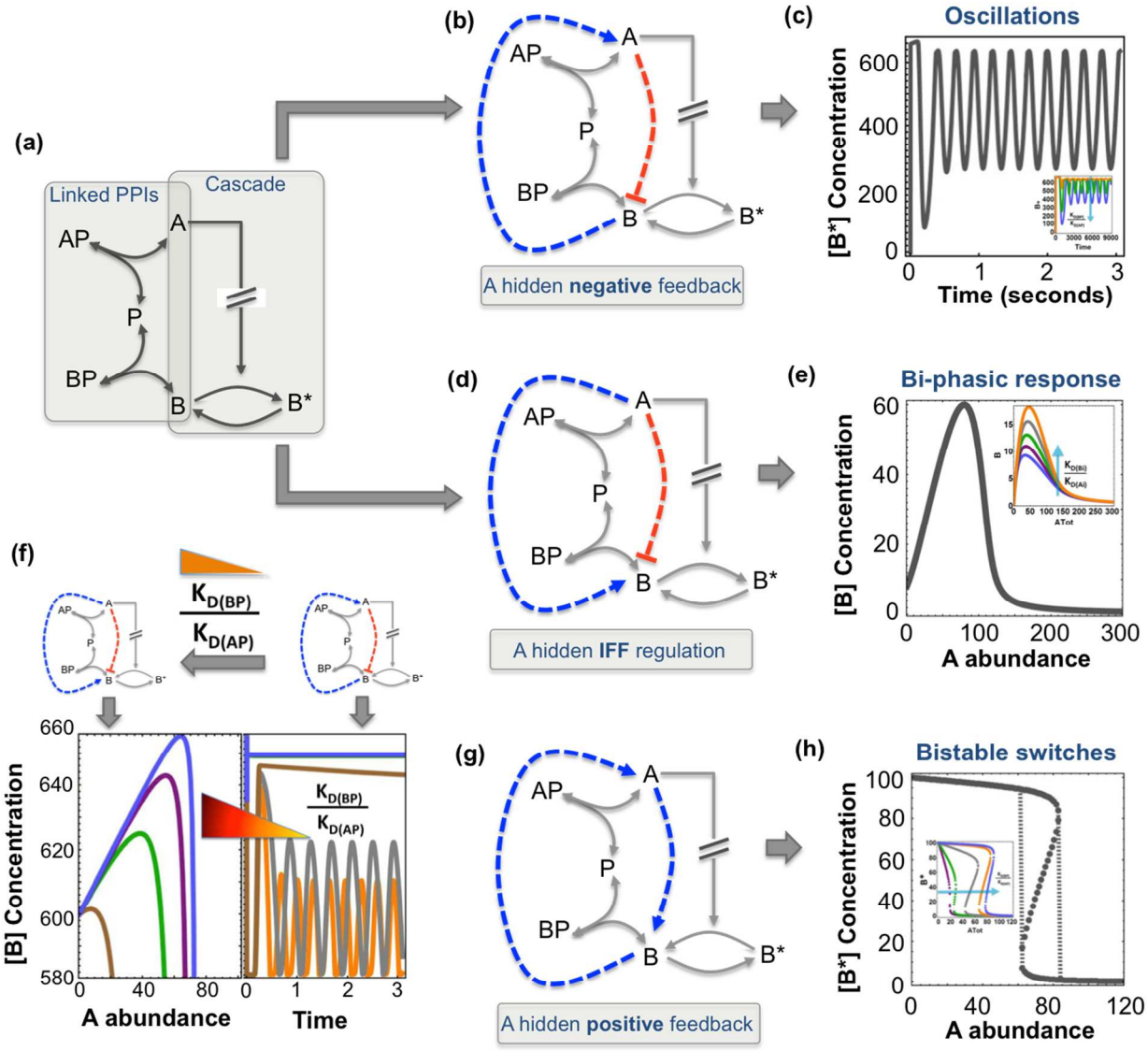

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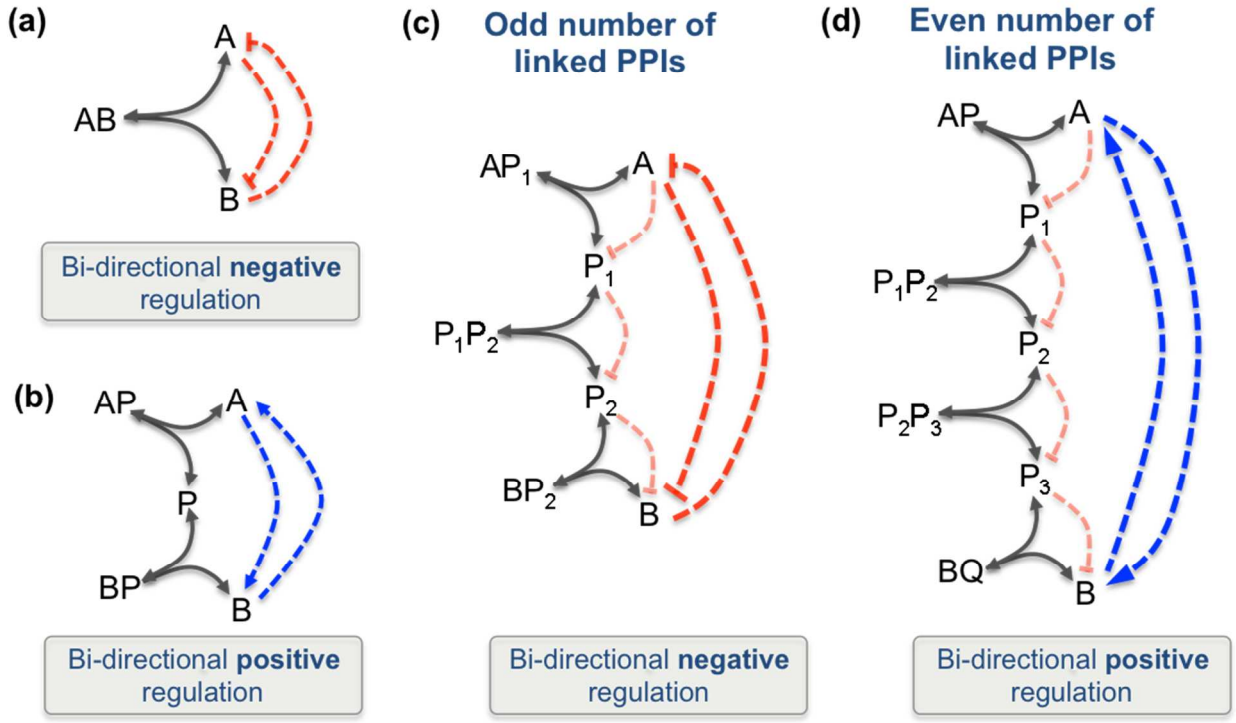

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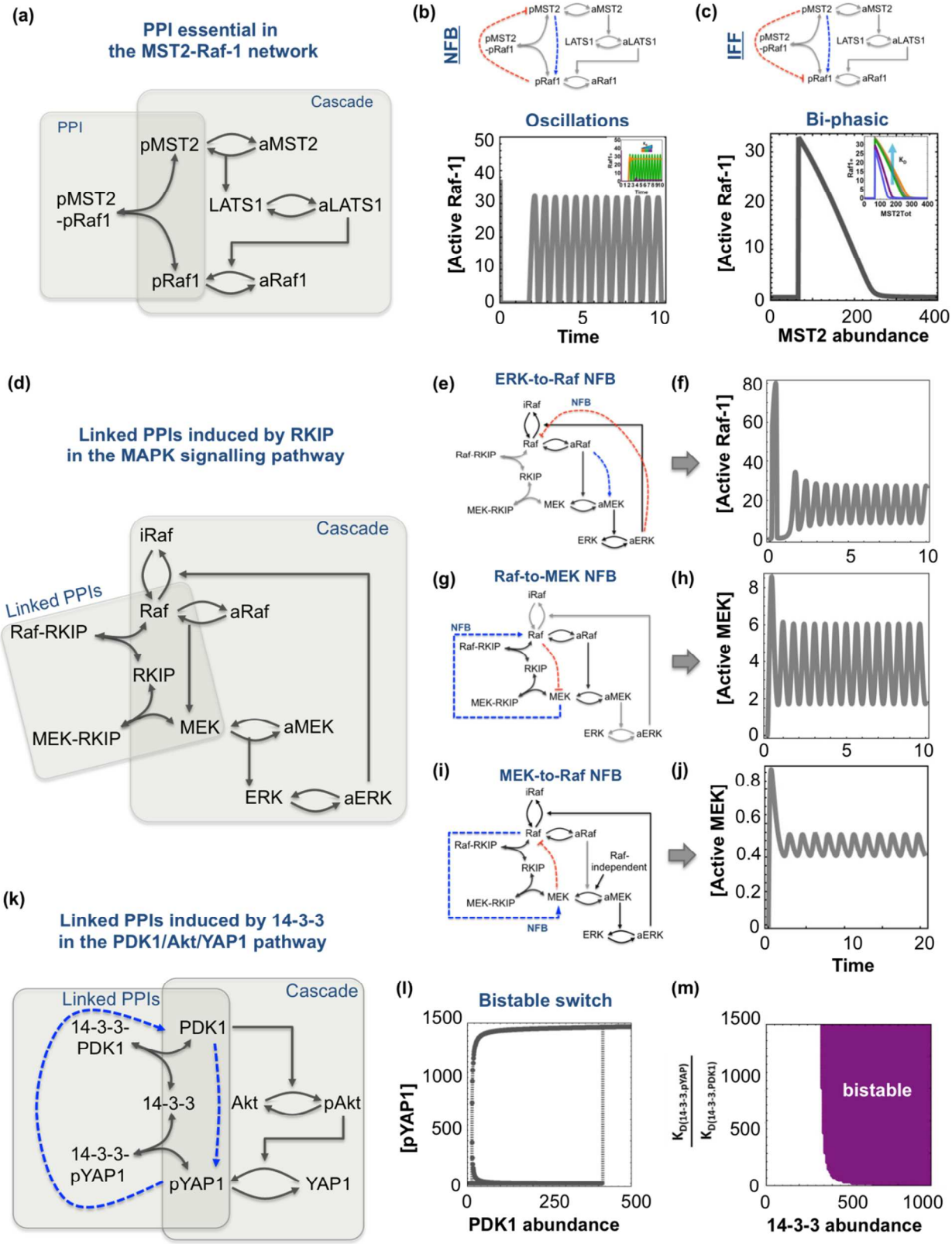

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