Feedback regulation in cell signalling: lessons for cancer therapeutics.

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Abbreviations

MST2: Mammalian STE20-like protein kinase 2
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
RKIP: Raf Kinase Inhibitor Protein
RSK: p90 Ribosomal S6 Kinase 2
IEGs: Immediate Early Genes
DUSPs: Dual Specificity Phosphatases
IRS: Insulin Receptor Substrates
mTORC1: Mammalian Target of Rapamycin Complex 1
Akt: Protein Kinase B (PKB)
PI3K: Phosphoinositide 3-kinase
FOXO: Forkhead box O
Gab1: GRB2-Associated Binding Protein 1
SOS: Son of Sevenless
Cdk: Cyclin-Dependen Kinase

Highlights

- Concept of feedback is fundamental for the understanding of signal transduction.
- Downstream protein that inhibits or activates upstream signalling protein(s) generates an explicit negative or positive feedback
- Hidden feedback is generated by a multitude of protein-protein interactions in signalling cascades
- Negative feedback brings about adaptation to external and internal cues, robustness to noise, dynamic plasticity and can also result in oscillations
- Positive feedback underlies robust dynamic and phenotypic switches in noisy environments
- Feedback regulation of signal transduction operates on different timescales and levels of organization
- Drug resistance often occurs as an intrinsic consequence of drug-induced perturbations to feedback regulation
- Design of new drug targets and effective drug combinations needs taking into account the feedback regulation on both short and long timescales.

Abstract

The notion of feedback is fundamental for understanding signal transduction networks. Feedback loops attenuate or amplify signals, change the network dynamics and modify the input-output relationships between the signal and the target. Negative feedback provides robustness to noise and adaptation to perturbations, but as a double-edged sword can prevent effective pathway inhibition by a drug. Positive feedback brings about switch-like network responses and can convert analog input signals into digital outputs, triggering cell fate decisions and phenotypic changes. We show how a multitude of protein-protein interactions create hidden feedback loops in signal transduction cascades. Drug treatments that interfere with feedback regulation can cause unexpected adverse effects. Combinatorial molecular interactions generated by pathway crosstalk and feedback loops often bypass the block caused by targeted therapies against oncogenic mutated kinases. We discuss mechanisms of drug resistance caused by network adaptations and suggest that development of effective drug combinations requires understanding of how feedback loops modulate drug responses.

1. Introduction

Living systems have acquired an impressive capacity to adapt to constantly challenging environmental conditions, while maintaining homeostasis. This dynamic plasticity is grounded in feedback control that operates at multiple levels of the cell and tissue organization. Not surprisingly, all cellular signal transduction networks are regulated by feedback loops. Sometimes, these feedback regulations are hidden within multitude of protein-protein interactions, as we will see below [1-5]. Feedback regulation comes in two essences, negative and positive feedback loops that endow network dynamics with different properties and dynamic features.

Negative feedback brings about robustness to noise and adaptation to perturbations within the feedback loop (for instance, caused by drug-induced inhibition of a protein inside the loop) [6, 7]. Yet, a strong negative feedback can also result in damped or sustained oscillations of both target and upstream proteins [8]. Positive feedback amplifies the signal and is often used by cells to convert analog input signals into digital outputs [9]. These digital outputs can involve a switch to a new phenotype when the analog input exceeds a certain threshold. Positive feedback can result not only in robust switches in noisy environments, underlying phenotypic transitions, but also in bistable responses to external and internal cues. For instance, several positive feedback loops in the retinoblastoma/E2F transcription factor system control the G1 to S-phase transition in the cell cycle [10, 11]. The positive feedback loops convert a gradual increase in growth factor concentrations into an “All-or-None” activation of E2F generating a bistable switch [12]. Bistability of the retinoblastoma/E2F system switch ensures that once switched ON, E2F activity remains high even in the absence of further growth factor signals, thus making the cell’s commitment to divide irreversible.

Feedback regulations of signal transduction operate on distinct timescales, precisely tuning the signalling outcome. When signals flow down through the network, immediate feedback regulations can occur as interactions of a target protein with an upstream protein and may also include post-translational modifications (PTMs), such as phosphorylation that alters the activity of the upstream protein. These immediate feedback loops operate on the timescale of seconds to several minutes, which is characteristic of protein interactions and PTMs. Feedback loops that occur through gene transcription and translation operate on longer timescales, starting from tens of minutes and persisting for several hours [13-17]. For instance, activated kinases such as the Extracellular Signal Regulated Kinase (ERK) and its downstream target, p90 Ribosomal S6 Kinase 2 (RSK), phosphorylate and activate transcription factors, thereby inducing transcription and translation of Immediate Early Genes (IEGs), such as Dual Specificity Phosphatases (DUSPs). IEG protein products can in turn feedback to the upstream signalling circuitry. For instance, ERK-induced DUSPs dephosphorylate ERK, suppressing ERK signalling [18]. Whereas feedback loops involving IEGs, such as DUSPs and cFos, can already be operational following 15 - 30 minutes from the signal onset, feedback loops incorporating Delayed Early Genes (DEGs) require about one hour or longer to generate an appreciable effect [19, 20]. Feedback loops that involve cascades of *de novo* expressed proteins are even slower, operating on timescales of several hours and days. Such delayed feedback regulations are likely a cause of long-lasting ERK activity pulses that persist over the course of four to five successive cell divisions [21].

The analysis of feedback control and crosstalk between signalling pathways is critical for successful drug treatment, since feedback loops and related pathway crosstalk dramatically change drug sensitivity and dose-responses. Drug resistance often occurs as an intrinsic consequence of network design features, and the analysis of feedback regulation can explain the disappointing clinical results. For instance, oncogenic BRAF(V600E) mutation is found in more than 60% melanomas and also in other cancers, including thyroid cancer and colorectal cancers. Although BRAF(V600E) is a driving mutation in these cancers, only melanoma patients show the initial remarkable response to specific RAF inhibitors, whereas colon and thyroid cancers are resistant to BRAF inhibition [22-25]. This resistance occurs because of activation of the Epidermal Growth Factor Receptor (EGFR) signalling by relieving the negative feedback from ERK to EGFR. Active ERK inhibits EGFR, but BRAF inhibition decreases ERK signalling, thus elevating EGFR signalling. This leads to activation of a multitude of EGFR targets, including increased RAS-GTP, CRAF, PI3K/AKT and mTOR signalling and alleviates the initial inhibition of the MAPK pathway, causing resistance to BRAF inhibitors. In contrast to colon and thyroid cancers, melanomas have very little EGFR expression, and thus are sensitive to BRAF inhibitors (before resistance inevitably emerges, usually after several months of the treatment [26]). Interestingly, overexpression of EGFR in melanoma cell lines renders these cells resistant to BRAF inhibitors, while colon cancer cell lines with low EGFR abundance were almost as sensitive as melanomas [23]. A combination of EGFR and BRAF inhibitors was shown to surmount resistance in colon cancer cell lines and mouse xenograft models of colon cancer [23, 24]. The discovery of new drug targets and design of new effective drug combinations need to account for the feedback and crosstalk structures of signal transduction networks. Until recently, the analysis of the effectiveness of drug combinations has mainly been left to empirical trial and error. Computational models of signalling networks explicitly include the network feedback control and crosstalk structures, predicting and explaining drug responses and emergent behaviours that are difficult to recognize experimentally or foresee intuitively.

Here we review roles of feedback controls in maintaining cellular homeostasis, while adapting to changing environments and drug perturbations. We discuss how negative feedback regulation provides systems robustness and positive feedback supports phenotypic switches by endowing signalling networks with ultrasensitive, switch-like responses and bistability. We show that this remarkable dynamic plasticity can also result from a multitude of protein-protein interactions that create hidden feedback loops in signal transduction cascades. We show how disappointing results in clinical trials of new drugs are rationalized by analysis of feedback regulations that are disturbed by these drugs within the same pathway or between different pathways. Finally, we suggest that computational models of feedback controls can be instrumental in predicting new drug targets and rational combinations of existing drugs.

2. Feedback control in maintenance of cellular homeostasis

2.1. Feedbacks as critical control mechanisms in signalling

Over the last several decades, an extensive body of work from theoretical, experimental and integrated studies have enriched and expanded our understanding of multiple roles of negative and positive feedback regulations. Notable features brought about by negative feedback include adaptation to continuously present cues and the ability to expand the linear range of steady-state input-output relationships, at the cost of decreased overall systems sensitivity to input signals [9, 27] . Negative feedback also brings enhanced robustness to the systems output against

perturbations occurring within the loop, a property that has its parallel in engineering devices (Fig. 1a) [1, 7, 28]. Dynamically, negative feedback enables adaptive and transient responses to sustained input signals [29, 30], and depending on the balance between the feedback strength and the time delay created by the feedback signal, negative feedback could bestow the system with damped or sustained oscillations (Fig. 1a), [8, 31]. Positive feedback can amplify input signals, thereby providing enhanced systems sensitivity [27, 32, 33]. Dynamically, positive feedback can shift a linear steady-state input-output relationship into a switch-like or even bistable profile, which is often exploited to create sharp transitions between downstream biological states in an all-or-none manner (Fig. 1b) [27, 34-38].

2.2. Non-redundant roles of multiple co-operating feedbacks

Although much has been learnt about the fundamental properties of negative and positive feedbacks when studied in isolation, many cellular systems have evolved not one but a multitude of feedback mechanisms, containing either negative, positive or mixed feedback structure. For examples, at least three different negative feedback loops regulate the oscillatory nucleo-cytoplasmic shuttling of the transcriptional nuclear factor kappaB [39]. Mixed positive and multiple negative feedbacks acting at different layers of the cascade have been described in the ERK pathway [40-42]. 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 [43, 44]. Given these widespread observations, it is intriguing to ask whether the combined feedbacks impart any functional advantages, and if so, how they are brought about by the feedbacks interplay.

Nested negative feedback is a recurrent design feature that is exploited by cellular systems. In the ERK pathway, ERK can phosphorylate and inactivate upstream regulators including RAFs [41], SOS [45], Gab1 [46], and the EGF receptor [47], creating nested negative feedback loops. Although their similarity in structural wiring suggests functional redundancy, model-based analytical and numerical analyses of various nested motifs showed that the individual feedback loops regulate oscillations in opposing manners [48]. While the long, outer loop promotes oscillations; the short inner loop suppresses oscillations instead. Such oscillations-inhibiting effect when strong enough can completely dominate the oscillation-inducing effect of the outer loop and destroy oscillations. Interestingly, altering the inner loop allows the system to widely modulate the period or amplitude of the oscillatory output, while keeping the mean signal value at near-constant levels. On the other hand, the outer loop allows the systems to flexibly modulate the oscillatory period while keeping the amplitude relatively stable [48]. These findings suggest that nested negative feedbacks provide the systems with robust ways to adjust different features of the oscillation dynamics, a property similar to that of the mixed positive–negative design [49]. Such ability to differentially tune oscillatory dynamics may contribute to the underlying reason why more than one negative feedback have evolved.

While negative feedback alone could induce oscillation, cellular oscillators often contain extra positive feedbacks. In these cases, the positive feedback acts like a modulator to systems dynamics, boosting robustness and enriching the repertoire of oscillatory patterns. Indeed, positive feedback combined with slow negative feedback can trigger relaxation oscillations where the system oscillates between the high and low branches of the hysteresis curve [50-52]. Positive feedback also reduces the nonlinearity required for a negative feedback to potentiate

oscillation, making it easier to achieve [53]. In the mammalian cell cycle, the presence of multiple positive feedbacks help amplify the amplitude of Cyclin-Dependen Kinase (Cdk) oscillation and enhance its robustness with respect to molecular noise [54]. In the same system, the interlinked feedback design also provides the oscillator with tunable frequency but nearly constant amplitude, a feature useful when adjustable frequency is non-essential [49]. On the other hand, interlocked positive-negative feedbacks were found to be critical in stabilizing the oscillation period in *Neurospora* circadian clock [55]. Together, these findings suggest that evolution has combined different feedback mechanisms not just accidentally, but cleverly to provide enhanced features to the cells for optimal fitness in the face of changing environments.

2.3. Hidden feedbacks arising from protein-protein interactions

Signal transduction occurs through protein-protein interactions (PPIs) and posttranslational modifications (PTMs). Interestingly, intricate dynamic behaviours, such as bistable switches or oscillations, can emerge from molecular interactions that create "hidden", or implicit, feedback loops in signalling cascades [4, 5]. In contrast to explicit feedback loops, which can readily be visualized on a graphical signal transduction map, hidden feedbacks challenge our visual intuition. One of the first interesting hidden feedback phenomenons was discovered a decade ago, showing the distributive double-site phosphorylation cycles of the MAPK single cascade can exhibit bistability without external positive feedback [4]. A key condition for bistability is the presence of competitive inhibition of at least one of the two opposing enzymes by the monophosphorylated intermediate, which functions like a positive feedback. Consequently, combining multi-site phosphorylation and a negative feedback could lead to relaxation oscillation [56], as seen in explicit positive-plus-negative feedback design. Moreover, hidden negative feedback is also observed in multi-tier signalling cascade, which can lead to sustained oscillation [57]. In this case, bistability at a single cascade is a prerequisite; and the hidden negative feedback effect arises from the sequestration of a kinase by its substrate at the next cascade level [33, 57].

We have recently reported additional mechanisms of hidden feedback loops that are attributed to simple PPIs [3]. Interactions between proteins that belong to different signalling cascade levels can lead to bistable switches, sustained and damped oscillations, and biphasic steady-state responses in various signalling networks in the absence of any external regulatory loops, as illustrated in Fig. 2. We extended our general analysis to physiological signalling systems including the MST2/Raf-1 crosstalk network and Raf-1/MEK/RKIP cascade that feature different PPI-related designs [3]. In the former system, interaction between the inactive forms of MST2 (Mammalian STE20-like Protein Kinase 2) and Raf-1 constitutes a single PPI motif whose binding affinity is strongly influenced by post-translational modifications of the interacting partners [58, 59]. When linked to the MST2/Lats1/Raf-1 cascade, this PPI forms a network design that could give rise to sustained oscillation and bisphasic dose-response. We have previously analysed the MST2-Raf-1 crosstalk and demonstrated theoretically and confirmed experimentally the presence of robust signalling switches [58, 59]. The predicted oscillation and biphasic response further suggest intriguing possibility for more complex physiological dynamics exhibited by this molecular circuitry, which demand future experimental verification. In the later system, the Raf Kinase Inhibitor Protein RKIP acts as a common endogenous inhibitor of both Raf-1 and MEK, which forms a coupled PPI motif design. While the classical, explicit negative feedback from ERK to Raf-1 could trigger oscillation; we showed

that the PPI structure independently bestows the system with oscillation [3], raising an interesting question as to how these two distinct oscillation-generating mechanisms may interplay (synergize or antagonize) under physiological contexts. Furthermore, due to the bi-directional nature of PPIs, PPI-containing networks such as those discussed could potentially exhibit co-existence of dynamics characteristic of either positive (negative) feedback or feed-forward regulation using the same network design. While explicit feedback and feed-forward loops are typically formed by post-translational modifications such as phosphorylation and ubiquitination [40, 59], these findings highlight that PPIs can result in hidden regulatory feedback and feed-forward loops. As PPIs are fundamental molecular events and central to most biological processes, their ubiquitous occurrence intriguingly suggests that hidden positive and negative feedbacks may be more widespread than previously expected.

3. Implications of feedbacks in cancer drug resistance

3.1. Emergence of drug resistance by feedback interference

The existence of multiple regulatory feedback loops is an evolutionary feature whose main role is to provide robustness to cellular behaviours in normal contexts. Ironically, what may have been essential for physiological functioning in many cases turned out to be stumbling blocks in combatting cancer. Multiple lines of evidence across different cellular contexts and cancer types have now revealed that feedback loops can be unexpectedly up-regulated or lost as side-effects of anti-cancer drugs. This either directly renders the drug ineffective or lead to activation of alternative pro-survival pathways, ultimately conferring resistance to the treatments. The lessons learned from these feedback regulations have been particularly useful in designing new therapeutic strategies where drug combinations, rather than single agents are used to hit the target and also to counterbalance the emerging changes in feedback controls.

3.2. Negative feedback loops tolerate inhibition of signalling proteins within a pathway.

An example of negative feedback implicated in drug resistance was reported by Sturm et al. who showed that inhibiting the MAPK/ERK pathway by MEK inhibitors is ineffective when negative feedback from ERK to RAF is strong [7]. Guided by modelling, the experimental work has demonstrated that due to negative feedback coupled with the inherent amplifier properties, the ERK pathway acts similar to a negative feedback amplifier (NFA), a design widely exploited in engineered devices to filter out perturbations to the NFA circuitry (Fig. 3a). MEK suppression induced by inhibitors is tolerated by the ERK pathway, as MEK inhibitors weaken the feedback signal and upregulate signal from RAF that compensates for the initial loss of ERK activity (Fig. 3b). This finding has two important implications for medicine: treatment strategies focused on inhibiting entities within NFAs should be cautiously avoided; and if not, they should be complemented with ways to break the feedback loop. The authors predicted and then confirmed *in vitro* that weakening the feedback with a RAF inhibitor will re-sensitize cells to MEK inhibitors [7]. This rather counterintuitive treatment regime receives further support from a recent study where siRNA screening of 37 KRAS mutant colorectal cancer cells showed RAF1 suppression was synthetic lethal with MEK inhibition [60], and a combination of MEK and RAF inhibitors is becoming a standard treatment in melanoma [61]. Consequently, targeting RAFs effectively reverse resistance to the MEK inhibitor selumetinib [60]. On the other hand, cells harbouring BRAF V600E mutations are devoid of NFA characteristics as BRAF V600E activity is feedback insensitive, rendering them sensitive to MEK inhibition [62].

The ERK-to-RAF feedback however, is not the only one that mediates drug resistance in EGFR signalling. Negative feedback from ERK to the EGF receptor (EGFR) has been implicated in the activation of parallel pathways including PI3K/Akt and reactivation of the MAPK pathway in response to RAF or MEK inhibition (Fig. 3c) [23, 63, 64]. While in physiological conditions, an ERK-to-EGFR feedback may serve to prevent over-activation of the receptor, this feedback is lost under RAF/MEK inhibition resulting in hyperactivated EGFR which triggers alternative signalling pathways and partially restores ERK signalling (Fig. 3d). As follows from the analysis above, combining RAF/MEK inhibitors with EGFR suppression that breaks the feedback loop to EGFR led to a synergistic inhibition effect [63, 64]. Importantly, the cross-activation of Akt by inhibition of the MAPK pathway components has been observed in many cancer types, including colorectal [23], gastric [65], melanoma [66], prostate [67] and breast cancer [68], suggesting a rather strong ERK-to-receptors feedback across different cancers.

Negative feedback has also been found to underlie resistance against drugs targeting the PI3K/Akt/mTOR pathway. In normal cells, stimulation of the insulin or insulin-like growth factor receptors activates insulin receptor substrates (IRS1/2) which trigger the downstream PI3K/Akt/mTORC1 cascade. A major substrate of mTORC1, S6K1, phosphorylates and inhibits the IRS proteins, inducing their degradation and abrogating their interactions with the receptors – generating a negative feedback loop from S6K1 to IRS1/2 (Fig. 3e) [69]. Since the PI3K/Akt/mTOR pathway is frequently hyperactivated in many cancers, multiple agents targeting PI3K, Akt and mTORC1 have been developed. However in many cases potent suppression of S6K1 by these agents leads to relief of the S6K1-mediated negative feedback. Loss of the feedback control causes increased IRS signalling and induction of the kinases upstream of S6K1, such as PI3K (in case of Akt inhibitors) and/or Akt (for mTORC1 inhibitors) which in turn activate the other, mTOR-independent pro-survival proteins (Fig. 3f,g) [70-72]. Interestingly, when PI3K itself is inhibited, triple-negative breast cancer cells break the old habit of upregulating PI3K and come up with a new way to circumvent drug effects. Instead of inducing activity of kinases within the feedback loop from S6K1, these cells exploit the feedback loss to stimulate insulin receptor (IR)/IGF-1R which in turn activates another pathway, the JAK/STAT [73, 74] and eventually reactivates PI3K/Akt (Fig. 3h) [75]. As in EGFR signalling, a dual-target treatment hitting both PI3K and JAK/STAT was shown to prevent resistance and curb tumour growth, further highlighting the importance of combinatorial therapies in these contexts [75].

3.3. Cross-pathway feedback loops

Drug resistance can be caused by feedbacks that operate within single pathways, e.g. the EGFR/MAPK and PI3K/Akt cascades, but also by feedbacks generated by crosstalk between different pathways. Rosen and colleagues have reported cross-pathway feedbacks that underlie resistance [76]. In a variety of tumour types, they found that PI3K/Akt inhibitors upregulate the activity and expression of several RTKs (including HER3, insulin and IGF-1R receptors), which re-stimulate PI3K/Akt signalling. The reactivation of these receptors is due to loss of negative feedback that forms between Akt and the RTKs via FOXO. Active RTK stimulates PI3K/Akt activity which in turn suppresses FOXO-dependent activation of the RTKs (Fig. 3i, j) [76]. While the recovery of PI3K/Akt signalling may be primarily a consequence of loss of the Akt-FOXO-RTKs feedback, it may also be attributed to the loss of the mTORC1-to-IRS negative

feedback discussed earlier. Using HER2-overexpressing breast cancer cells, the same group found that PI3K inhibition induced HER3 activation results in enhanced ERK instead of PI3K/Akt signalling [77]. In the case where PI3K/Akt signalling is reactivated, combined inhibition of Akt and HER3 kinase activity yielded synergistic effects; while for reactivation of the EKR signalling, combining PI3K with MEK inhibitors led to enhanced anti-tumour activity compared to administration of single agents.

The importance of the Akt-FOXO-RTKs feedback in drug resistance is further corroborated by recent data in pancreatic cancer [78]. In this study, Wei et al. reported that AZD8055, an ATP-competitive inhibitor of mTOR, failed to robustly inhibit pancreatic cancer cell growth. Although AZD8055 initially efficiently suppresses mTORC1/2 and Akt activity, such suppression is only transient for Akt. Subsequently, Akt reactivation ensues due to FOXO-mediated upregulation of the EGFR receptor. The authors concluded that EGFR-induced reactivation of Akt and other downstream kinases, such as ERK, contribute to AZD8055 resistance. Consequently, concurrent inhibition of mTOR and ERGF signalling using AZD8055 and erlotinib significantly sensitizes pancreatic cancer cells to AZD8055 both in vitro and in vivo, suggesting this combination as a potential novel treatment strategy for pancreatic cancer [78].

Combination therapies are also proved to provide better efficacies in other cases of drug-induced cross-pathway activation mediated by positive and negative regulatory feedbacks. In lung cancer patients, poor responses to cetuximab, a specific EGFR monoclonal antibody inhibitor, observed in several clinical trials [79] is attributed to the compensatory upregulation of HER2, HER3 and HER3-MET interactions, which lead to consequent reactivation of EGFR and ERK signalling [80]. Overcoming the feedback-induced effects required a triple combination of antibodies targeting the EGFR, HER2 and HER3 receptors [80]. In non-small cell lung cancer, feedback upregulation of IL-6 and STAT3 drives resistance to RTK/MEK inhibitors [81]. This finding provides a rationale for combining RTK/MEK inhibitors and those of STAT3 and/or its upstream kinases for this lung cancer subtype. In a similar vein, IL-6 was found markedly upregulated in gastric cancer cells when treated with prolonged exposure to HER2 inhibitor, trastuzumab [82]. In addition to the IL-6-mediated STAT3 activation, resistance to trastuzumab was also attributed to Notch activation, which suggests the STAT3/Notch signalling axis as a potentially useful therapeutic target in gastric cancer [82].

4. Computational modelling as a valuable tool for therapeutic research

Although efforts in mathematical modelling and computational simulations to dissect the functional roles of feedback loops in shaping systems dynamics have been extensive, their application as a tool for feedback-related therapeutic research remains modest [33, 40, 83, 84]. Mechanistic signalling network models can provide an in-depth understanding of feedback-dependent drug resistance and facilitate the development of novel therapies. The models not only aid in providing solid explanation for experimental data, but also help to identify suitable targets and predict effective combinations of drug treatments.

As an illustrative example, we constructed a simplified mathematical model of the Akt/mTOR and RTK/MAPK pathway crosstalk, which incorporates the key feedbacks that have been implicated in drug resistance, as discussed in the previous sections (Fig.4a). Here we show that despite its simplicity and high-level abstraction, this model can recapitulate existing

experimental observations and generate testable predictions. As detailed in the reaction scheme in Fig.4b, the model describes the activation of the ERK/MAPK pathway by the ErbB family receptor and activation of the PI3K/Akt/mTORC1/S6K pathway by IR/IGFR. For simplicity, the model assumes a single input for each pathway (Fig. 4b). The negative feedbacks from ERK to Raf and the RTKs, and from S6K to IRS are included. In addition, the cross-pathway feedback from Akt to the RTK via FOXO proteins is incorporated in the model. The model also includes generic reactions of inhibiting PI3K, Akt and MEK using their specific inhibitors (see reactions shown in red in Fig.4b). The model is formulated using mass-action kinetics and ordinary differential equations (all model equations and parameter values are given in the Supplementary Information (SI)).

We first simulate the effects of Akt inhibition on the systems dynamics focusing on the levels of active ERK and S6K that are key signalling outputs of the network. Time-course simulations displayed in Fig.4c show that under increasing Akt inhibition, mTORC1 activity is potently suppressed, evident by the proportionally inhibited level of active S6K (left panel). On the other hand, model simulations demonstrate that PI3K and ERK activity increase with the increasing AKT inhibitor dose, which is not immediately expected (middle & right panels). The enhancement of PI3K and ERK activities by AKT inhibitor is explained by drug suppression of the S6K-to-IRS and the Akt-FOXO-RTKs negative feedback loops. In fact, shutting down these regulatory links abrogate the dependence of PI3K and ERK activity on Akt inhibitors. Thus, despite its simplified form, this model is capable of qualitatively recapitulating the major experimental observations discussed in the previous sections.

The model is not only useful in providing mechanistic explanations of the observed experimental data on resistance against PI3K/Akt inhibitors and generate valuable insights into how the system rewires under various perturbed conditions, it could also be used to make new testable predictions. For example, the model predicts that in cells where the Akt-FOXO-RTKs negative feedback is operational, upregulation of FOXO expression strongly influences the quantitative effects induced by Akt inhibition. As shown in the steady-state dose-response simulations in Fig.4d, higher FOXO expression results in the stronger enhancement of PI3K and ERK activation by the Akt inhibitor, particularly at higher levels of inhibitor dosage (red vs. black curves). Thus, the degree of switching on of PI3K and ERK signalling due to Akt inhibition may be markedly different in different cell types that express different levels of FOXO abundance. Consequently, epigenetic or genetic alterations resulting in changes of FOXO expression may influence the degree of resistance to Akt inhibitors mediated via the Akt-FOXO-RTK negative feedback. These predictions form testable hypotheses that would be interesting to experimentally verify.

In addition, the model can further make predictions of responses to MEK inhibition, which is commonly used therapeutically to block ERK signalling in many cancers. Interestingly, although MEK inhibition effectively shuts down ERK signalling (right panel, Fig.4e), it is predicted to promote both mTORC1 (left panel) and PI3K/Akt (middle panel) signalling activation under a strong and operational ERK-to-RTK feedback. Indeed, MEK inhibition leads to the loss of the ERT-to-RTK feedback resulting in RTK upregulation and thus compensatory PI3K/Akt and S6K signalling. The model predicts that increasing MEK inhibition at low inhibitor dosage strongly affects ERK and S6K activities, but only slightly influences PI3K activity (Fig.4f). On the other

hand, increasing MEK inhibition at high inhibitor dosage exerts opposite effects, resulting in steep activation of PI3K accompanied by only slight changes in S6K and ERK activation (Fig.4f).

5. Perspective and conclusion

We showed in this review that drug-induced up- and down-regulation of feedback loops dramatically changes responses to these drugs, often leading to unanticipated drug resistances. These effects can only be understood at the network level aided by computational models of these networks. We evidenced remarkable effects of the difference in protein abundances and therefore, feedback strengths on responses to drugs and their combinations, as was illustrated by considering the different FOXO abundances in the model. We conclude that quantitative understanding of drug-induced signalling effects and genotyping tumors is an absolute necessity before using these drugs in patients.

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

Figure legends

Figure 1. Properties of negative and positive feedback regulations. (**a)** Negative feedback loops could: expand the input/output operating linear range; tolerate perturbations within the loop; enable transient/adaptive dynamic response; and induce damped as well as sustained oscillations. **(b)** Positive feedback loops could: induce switch-like time-course dynamics; amplify input signal; generate switch-like and bistable steady-state dose-response dynamics where the system could reach either a low or high steady state depending on its past state.

Figure 2. PPIs generate hidden feedback/feed-forward loops and complex behaviours. (a) A single PPI linking unmodified components belonging to different layers of a cascade could induce bistable switches (modified moieties are denoted with a star, hereafter), caused by a hidden double-negative feedback illustrated in the right panel. **(b)** A PPI linking unmodified/modified cascade components could induce sustained oscillation, triggered by a hidden negative feedback depicted in the right panel. **(c)** Coupled PPIs motif linking unmodified forms of the cascade components could generate oscillation and biphasic dose-response, brought about by a hidden negative feedback or a incoherent feed-forward (IFF) loop (right panel).

Figure 3. Drug resistance mediated by feedback interference. (a, b) Suppressing MEK with a specific inhibitor (In) breaks the ERK-to-Raf negative feedback loop, leading to re-activation of ERK (active species are indicated by red star). **(c, d)** MEK inhibition breaks the ERK-to-EGFR negative feedback loop and stimulates an alternative (PI3K/Akt) pathway. **(e)** Schematic diagram of an intact S6K-to-IRS negative feedback. Specific inhibition of mTORC1 **(f)**, Akt **(g)** or PI3K **(h)** all break the S6K-to-IRS negative feedback, but lead to either Akt activation **(f)**, PI3K activation **(g)** or activation of the JAK/STAT pathway **(h)**. **(i)** Crosstalk between the IR(IGFR)Akt/mTORC1 and RTKs/RAF/MEK/ERK pathway mediated via a negative feedback loop involving FOXO. **(j)** Akt inhibition breaks the S6K-to-IRS negative feedback and de-suppresses FOXO leading to activation of PI3K and RTKs/ERK signalling.

Figure 4. Computational model of the IR(IGFR)/mTORC1 and RTKs/ERK pathway crosstalk and model predictions. (a) Schematic diagram of the pathway crosstalk including the major negative feedbacks. **(b)** Reaction scheme of the model, based on which differential ordinary equations (ODEs) are formulated (model description is given in the SI). **(c)** Time-course simulations of active S6K, PI3K and ERK levels for increasing Akt inhibition (time unit is in minutes). **(d)** Steady-state simulations of active S6K, PI3K and ERK levels in response to increasing dose of the Akt inhibitor (AktI total) for high/low FOXO expression. **(e)** Time-course simulations of active S6K, PI3K and ERK levels for increasing MEK inhibition. **(d)** Steady-state simulations of active S6K, PI3K and ERK levels in response to increasing dose of the MEK inhibitor (MEKI total). Parameters used for plotting are given in the SI, Table S1 and S2.

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