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REVIEW

Robustness of signal transduction pathways

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Abstract Signal transduction pathways transduce information about the outside of the cell to the nucleus, regulating gene expression and cell fate. To reliably inform the cell about its surroundings, information transfer has to be robust against typical perturbation that a cell experiences. Robustness of several mammalian signaling pathways has been studied recently by quantitative experimentation and using mathematical modeling. Here, we review these studies, and describe the emerging concepts of robustness and the underlying mechanisms.

Keywords Robustness · Feedback · Signal transduction · Adaptation · Sensitivity · Heterogeneity · MAPK · BMP

Introduction

Systems that remain functional although they experience a perturbation are termed robust [1]. Robustness is a central feature of living systems, as these systems face many strong perturbations on all levels [2]. Perturbations range from changing environmental conditions in which an organism lives, wounds and infections that act on the tissue level down to perturbation of the cellular state due to fluctuation in supply of nutrients or stochasticity and uncertainty in molecular processes [3]. Robustness is typically limited to moderate perturbation magnitudes, and can only be maintained for certain molecular species and dynamical properties of the network. Quantitative perturbation-response measurements are thus required to pin down precisely which functions a system can maintain. Recently, robustness of signaling pathways has been investigated by several groups. These studies employed theoretical concepts such as parameter optimization and sensitivity analysis to identify robustness-promoting mechanisms, and to derive experimentally testable predictions. Here, we review these findings, discuss how robustness can be investigated, and how it is implemented in signaling pathways.

Types of biological robustness in signaling pathways

The function of signaling pathways is to transduce information about the outside of the cell and the cellular state to the nucleus, where they ultimately inform and regulate the expression of genetic material. The mode by which signal transduction pathways transduce information may be very different for different pathways. Some signaling pathways are thought to faithfully report the concentrations of ligands at the outside of the cell to the nucleus, thus transducing quantitative information about the extracellular milieu. Other signaling cascades are thought to work binary, thus essentially reporting whether a stimulus is present or not. Signaling pathways may also display distinct dynamic patterns (e.g., oscillation frequencies) that contain information about the type or concentration of ligand [4, 5].

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In order to disentangle which quantitative feature of signaling pathways (e.g., duration, frequency, amplitude) is important, it is useful to quantify information transmission in single cells, and then apply information theoretical approaches [6]. Such approaches allow selecting the feature of the signal that correlates best with cellular outcome or the stimulus. These may also be the biologically most meaningful features. To disentangle quantitative and binary signaling, the sigmoidality of the input–output relation can be quantified using measures like the Hill coefficient [7].

Depending on the type of signaling pathway, robustness may refer to different aspects of the response: for signaling pathways that transduce information quantitatively, robustness may refer to an invariance of the absolute signal level. Alternatively, robust information may be encoded in the fold-change in the signal depending on the stimulus strength [8, 9]. Then the absolute level may be very variable, but the fold-change faithfully reports the stimulus strength. Here, downstream networks have to translate fold-changes back into meaningful biological signals. For pathways that report binary whether a signal is present, it may be most crucial that no signal is transmitted if no ligand is present, and that the pathway is activated upon strong stimulation. Moreover, binary cell fate decisions may require that the dose–response of signal transduction is always sufficiently steep [10]. Whether the threshold at which cells respond should be a robust property may depend on the biological system: for example, a moderate apoptosis trigger should kill a proportion of the cells, not the whole population. Thus, single cells in a population should differ in their apoptosis thresholds [11]. Likewise, it has been argued that imprecise decision-making in neuronal differentiation ensures that only a subset of the population enters the terminal stage, while the remainder continues to proliferate [12]. Other examples of tissue-level systems in which it is beneficial that only a subset of cells responds is the immune system (see for example [13] for the stochastic origin of heterogeneity in Th2 differentiation), or the response to TNF-α [14]. Robustness of signaling thresholds is, however, expected to be important in embryonic development: according to the so-called "French-flag model" [15], patterning is established by a single morphogen gradient that specifies multiple cell fates, each cell type requiring a different threshold morphogen concentration. For sharp spatial boundaries to be established, signaling pathways that read of morphogen gradients should exhibit robust and invariant thresholds at which they respond. For example, it has been argued that intercellular communication by the Notch–Delta system and self-organizing morphogen gradients may be important for robust patterning [16, 17].

Biological systems are subject to internal and external fluctuations, all of which may result in non-robust behavior. The dynamics of regulatory networks operating at low molecule numbers may exhibit stochastic dynamics, and will thus show intrinsic variability. However, most signaling pathways operate at a fast time scale compared to slow downstream cell fate decision-making. Thus, we do not expect that the stochastic dynamics intrinsic to signaling systems impinge significantly on phenotypic responses [18]. Nevertheless, signaling pathways can show non-genetic cell-to-cell variability [11]. Biological mechanisms underlying variability include cell density effects [19], and cell-to-cell variability in signaling protein expression [8, 20]. In the latter case, stochasticity in protein biosynthesis indirectly hampers precise cellular decision-making [21]. Even if non-genetic variability among cells is negligible, we expect that biological networks should be able to buffer more homogenous perturbations such as temperature variation and germline mutations. Fluctuations may also occur on a multicellular scale, e.g., during embryonic development: individual embryos will have different size depending on internal and external conditions, but the proportions of embryonic patterns nevertheless need to be maintained. Remarkably, this so-called "embryonic scaling" can be established by the self-organizing properties of morphogen gradients [22]. A fascinating example for robust self-organization is the so-called Spemann organizer: this small patch of cells can establish a complete Siamese twin when grafted onto another embryo [23, 24].

In the following, we review the different types of robustness that have been observed in signal transduction. We focus on concepts and tools that have been developed using mathematical modeling approaches. We discuss which network design principles facilitate robust information processing and show how synthetic biology approaches contributed to our understanding of robustness. Finally, we review recent research that has identified negative feedbacks as a source of robustness in MAPK signaling and BMP signaling.

Mathematical concepts

Robustness refers to insensitivity towards perturbations. Such perturbations may arise intrinsically from the stochastic dynamics of the system, or may originate from extrinsic mechanisms (e.g., variability in signaling protein expression). Stochastic simulations algorithms would be required to simulate variability intrinsic to signaling processes. For reasons discussed in the previous section, we will neglect stochasticity in signal transduction and will thus focus on deterministic modeling approaches based on ordinary differential equations.

In mathematical terms, insensitivity against extrinsic perturbations is typically analyzed using a normalized slope, the so-called logarithmic gain [25].

$$G = \frac{d \ln R}{d \ln p} = \frac{p}{R}\frac{dR}{dp} \quad (1)$$

Here p refers to a perturbation of the system (e.g., a change in a protein concentration), while R denotes the biological response of interest. The logarithmic gain is a dimensionless number that quantifies how a relative change in the perturbation translates into a relative change in the response. For example, a logarithmic gain of unity refers to a linear relationship between perturbation and response, while values of for example ½ and 2 imply sub and supra-linear behavior, respectively. Systems with logarithmic gains smaller than unity are frequently termed (partially) robust or subsensitive, whereas the term ultrasensitivity is used if logarithmic gains exceed unity. In metabolic control theory, the logarithmic gain is known as the control coefficient or response coefficient, depending on the context [26]. Summation theorems of metabolic theory reveal that control coefficients of biochemical networks generally sum up to a fixed number, known as total control [27, 28]. In terms of robustness, summation theorems imply that a biochemical network can never be insensitive against all possible perturbations. The term of the "robust yet fragile nature of biological networks" was coined to describe that robust systems will always be highly sensitive towards certain perturbations [29]. As further discussed below, this trade-off in the evolution of robustness can be circumvented and global robustness be realized in living cells if perturbations are coupled, e.g., by co-regulating enzymes controlling the antagonistic reaction steps.

A drawback of logarithmic gains is that they quantify sensitivities locally: by definition, Eq. (1) considers only infinitesimal small perturbation strengths and thus does not contain information about robustness against stronger perturbations. Effects of larger parameter changes have been analyzed, e.g., by considering higher-order derivatives [30] or rounding parameter values to the next order of magnitude [31]. Moreover, logarithmic gains are valid only for certain combinations of kinetic parameters unless analytical expressions can be derived. Global sensitivity analysis tools have been developed to determine numerically whether a network shows structural robustness irrespective of the particular choice of parameters. Typically, global sensitivity analysis relies on systematic sampling of the parameter space and statistical evaluations of parameter sensitivity distributions. These sampling strategies may, however, consider many physiologically irrelevant parameter sets, thus missing essential biological constraints and give rise to misleading results. To resolve this problem, sensitivity analysis should be restricted to parameter regions compliant with available experimental data, e.g., by applying parameter identifiability analysis tools [32]. Another important aspect of robustness in signaling networks was addressed in two recent publications [33, 34]: while sensitivity analysis is typically restricted to a single operation point of the system (e.g., a particular steady state), it may be important for signaling networks to maintain the complete dose–response curve invariant despite molecular perturbations. Shinar et al. [34] and Steuer et al. [33] showed that certain network topologies can show invariance of all positive steady states, and termed this phenomenon absolute concentration robustness.

We conclude that biologically meaningful robustness analyses using logarithmic gains require systematic investigations of larger parameter regions. Additionally, a careful definition of the biological readout of interest is necessary, in particular, if time-dependent processes are considered. Recently, an elegant study applied time-dependent sensitivity analysis using Lyapunov exponents to obtain insights into the dynamics of apoptosis initiation [35]. In the following, we will discuss biochemical network motifs that respond subsensitively to perturbations, and thus confer robustness to biological regulation.

Robustness-promoting network motifs: evidence from modeling and experimentation

Biochemical networks are characterized by recurrent motifs, each of which fulfils a spectrum of dynamical functions, and can thus be considered as a modular building block for biological regulation [36, 37]. Several of these motifs have been implicated in promoting robustness including negative feedback, incoherent feed-forward loops, and functional redundancy. In the next paragraphs, we will review theoretical and experimental evidence supporting their role in robustness.

Incoherent feed-forward loop

One of the simplest motifs that can provide robustness is an incoherent feed-forward loop (Fig. 1a): a signaling molecule activates a target by one route, and at the same time it de-activates the target by another route. Thereby, an increase in the concentration of the signaling molecule leads to both an increase and a decrease of the activity of the target, which can then cancel out. To what extent such incoherent feed-forward loops provide robustness depends crucially on the regulatory strength of both routes. If for example the deactivating route is weaker, it cannot fully compensate an increase due to the activating loop, and the system would only be partially robust. On the other hand, if both routes have the same strength, they can cancel each other, and the system would be fully robust. Then,

however, a change in the signal would have no consequences on the target activity, and information could only be transmitted if both routes have different kinetics. If for example the activating route is faster, the target would be activated, and subsequently the slower deactivating route would reset the target to a robust steady-state activity. Using synthetic biology approaches, robustness mediated by feed-forward loops has been quantitatively analyzed in transcriptional regulatory networks, both experimentally and theoretically [38].

Robustness due to incoherent feed-forwarding is somehow optimized in many two-component signaling pathways in bacteria, which are built of a histidine kinase that activates a response regulator by phosphorylation: in many two-component systems, the histidine kinase is a bifunctional enzyme that apart from phosphorylating the response regulator also acts as the phosphatase of the response regulator. Thus, the histidine kinase can be considered to control the response regulator by an incoherent feed-forward mechanism (Fig. 1b). This can lead to perfect concentration robustness where the phosphorylation of the response regulator is independent of the concentration of the kinase [39].

In other bacterial signal transduction systems, enzymes that perform opposing biochemical functions in signaling pathways, such as kinase and phosphatase, or methyltransferase and methylesterase, are expressed from the same operon (Fig. 1c). Thus, an increase in expression of that operon due to gene expression noise leads to an increase of both activating and deactivating signals, which can then be canceled out. This correlated fluctuation strategy thus provides concentration robustness against variability of pathway components. A prime example is bacterial chemotaxis, where for example the methyltransferase and methylesterase of the receptor are encoded on the same operon [40]. The robustness of this pathway is further optimized by coordinated translation of pathway components: genes whose protein products need to be highly correlated are adjacent on the operons. This establishes translational coupling, which further improves correlated protein expression and concentration robustness [41].

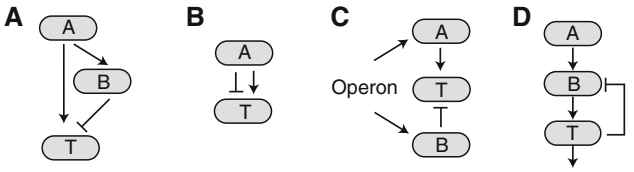

Fig. 1 Network motifs that cause robustness in signal transduction. **a** Incoherent feed-forward loop. *A* activates the target *T* directly, and deactivates it indirectly via *B*. **b** The bifunctional enzyme *A* activates and deactivates *T* (possibly depending on its phosphorylation state). **c** *A* and *B* exert opposing regulation on *T*, and are expressed from the same operon. **d** *A* activates the target *T* via *B*, and the target activity feeds back into the signaling pathway by negative feedback regulation

Negative feedback (Fig. 1d)

It has long been known in the engineering sciences that robustness can be established if the system output feeds back to the input negatively and thereby corrects the upstream control unit. A well-known example of such negative feedback regulation is a thermostat controlling a heater: if the temperature becomes too high, the temperature control device switches off the heater, thus shifting the system towards the desired temperature. Feedbacks can be of different types: a feedback may depend on the present output (called proportional feedback), it may depend on the temporal change (which is called differential feedback, and essentially aims to predict the future), or an integral over the past (integral feedback). Engineered devices often employ a mixture of these, which are called PID (proportional—integral—differential) controllers. While proportional and differential feedback can only provide partial robustness, integral feedback can fully buffer variations. This can be explained as follows: the function of feedback is to measure the output and to correct for deviations by adjusting upstream cascade levels. A drawback of proportional and differential feedback is that perturbations are estimated at the present time only. Thus, deviations at the output level can never be fully compensated, because the feedback becomes very weak before the output is fully adapted. In contrast, an integral feedback can memorize from the past how much adjustment is needed to correct for perturbations. Thus, integral feedback can remain strong until the output is fully compensated.

In yeast osmoregulation, negative feedback regulation of the HOG1-MAPK signaling pathway leads to robustness and helps to perfectly adapt to different osmolarity, and the mechanism could be traced back to an integral feedback [42]. A further very well studied example for integral feedback is bacterial chemotaxis. Due to the integral feedback, the steady-state level of the response regulator is independent of several parameters, rendering the system robust against many types of variations [43]. Often, integral feedback is difficult to be identified, as the integral is distributed on many components, and requires complicated mathematical formalisms, such as those presented in [33].

Negative feedback provides robustness at a cost: it reduces the sensitivity of the system to signals. Thus, the signal is dampened by the feedback, and the signaling pathway itself may need to strongly amplify the signal to be capable of transducing information in the presence of feedback. This trade-off can be very well analyzed using response analysis, which has been developed for biological systems by Kholodenko and coworkers [44–46]. Using response analysis, the

pathway response to external stimulation can be expressed as a function of the feedback strength.

$$R = r/(1 - r * f) \quad (2)$$

Here, the logarithmic gains r and R quantify how the pathway activity is affected by changes in the external stimulus (defined similarly to Eq. 1). The logarithmic gain r characterizes the pathway response in the absence of feedback, while R additionally takes into account negative feedback regulation. Equation (2) thus reveals that negative feedback always reduces the gain of the pathway ($R < r$). The feedback strength parameter f quantifies how strongly the downstream species in the signaling pathway affect the activity of upstream intermediates (note that $f < 0$). Increased negative feedback strength will result in larger negative numbers of $r*f$, thus decreasing pathway responsiveness to external stimulation. Note that for strong feedback in an ultrasensitive multi-level signaling cascade, oscillations tend to arise [47].

Negative feedback is widespread in transcriptional networks, both in bacteria [48] and in mammals [49]. The function of such negative autoregulation of transcription factors is believed to lie in decreasing response time [50], but also in mediating robustness to biochemical fluctuations [51, 52] and genetic mutations [53]. Also, virtually all signaling pathways in mammals are equipped with transcriptional feedback, where a signal causes the transcriptional up-regulation of inhibitor proteins [54]. These proteins utilize a wide variety of biochemical functions by which they inhibit signaling. They include phosphatases (e.g., DUSPs in MAPK signaling [55, 56], PHLPP in Akt signaling, phosho-diesterases (PDE4 in cAMP signaling), stoichiometric inhibitors (SnoN and Sprouty), as well as proteins regulating ubiquitin ligases (SMAD7 and SOCS2). The proteins engaged in negative transcriptional feedback have the interesting property that they are generally very short lived, allowing them to respond rapidly towards stimulation [54]. Transcriptional feedback has been predicted by computational studies to mediate robustness in target gene expression, mainly by compensating heterogeneity in transcription rates of their targets [57].

In the following, we will review two combined experimental theoretical studies that our groups have recently conducted that address the roles of negative feedback that act in signaling on post-transcriptional level [58] and by transcriptional induction of negative regulators [59].

Robustness against protein concentrations in MAPK signaling

MAPK signaling is a three-tiered signaling cascade that is highly conserved amongst eukaryotes, and is involved in the control of many cellular processes and decisions. The classical MAPK cascade consists of the kinases RAF, MEK, and ERK, and is mainly activated by a small GTPase called RAS. Activation within the pathway is due to phosphorylation of the kinases (see Fig. 2a). The mode of information processing varies fundamentally between cell types, stimulus and organisms, ranging from essentially binary signaling [7] to nearly linear information transmission in the control of cell growth in tumor cell lines, at least on the population level [60]. The mode of operation is possibly determined by feedback or feed-forward loops [61, 62]. Recent live-cell imaging has unveiled that protein expression of the terminal kinases, ERK, is varying with a standard-deviation of 20–30 % in single cells [8]. Thus, the question arises as to how this signaling pathway can reliably transmit quantitative signals, when the components vary so strongly.

ERK is activated by reversible phosphorylation. A theoretical kinetic analysis shows that a change in ERK protein level will linearly translate in a change in phosphorylated, activated ERK. For example, if the protein level is reduced by 50 %, a decrease of 50 % in the amount of phosphorylated protein is predicted to occur. Thus, the logarithmic gain equals one, and covalent modification cycles are predicted to be non-robust against varying protein levels. Consequently, we expect that cells that receive a defined stimulus would nevertheless display very variable ERK activity due to variable ERK concentrations (see Fig. 2b, left branch). In contrast, if signaling shows some robustness and phosphorylation is only sub-linearly dependent on ERK levels, ERK activity would be much more confined (see Fig. 2b, right branch).

To investigate whether ERK signaling is robust against protein variations, we systematically lowered ERK concentration using siRNAs against the two isoforms of ERK, and quantified ERK protein levels and ERK phosphorylation [58]. We find that ERK in colon cancer cells is robust, i.e., its logarithmic gain is smaller than one. Reduction of ERK levels down by 80 % resulted in reduction of ERK phosphorylation by about 40 %. This raised the question of which mechanism would be responsible for this robustness. After experimental evidence excluded kinetic effects at the level of the core MAPK cascade (cf. [63]), we speculated that negative feedback may be the mechanism by which robustness is generated.

The signaling pathway is known to be strongly feedback controlled, both by transcriptional [54, 56] and post-translational feedbacks [64]. To our surprise, transcriptional feedback via dual-specificity phosphatases (DUSPs) could be excluded, as they did not respond when ERK levels were changed [63].

Thus, our main hypothesis of why this pathway is robust was post-translational feedback. As there are multiple

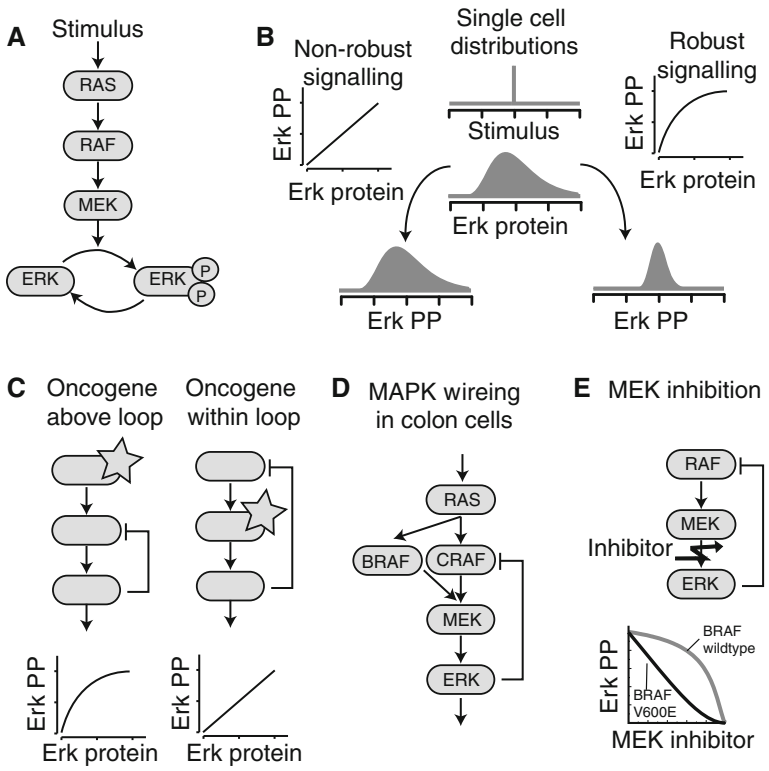

Fig. 2 Robustness in MAPK signaling. **a** Schematic representation of the classical MAPK signaling cascade, triggered by RAS, involving the kinases RAF, MEK, and converging at the kinase ERK which has to be double-phosphorylated to become active. **b** If the protein abundance in cells (exemplified by ERK) varies strongly between cells, a confined stimulus given to these cells will result in heterogeneous response when signaling is not robust (*left branch*), or to a more confined response, if signaling is robust, i.e., when the signal depends only sublinearly to the concentration of the protein. **c** Strategy to dissect the role of robustness due to feedback by oncogenes. If a feedback causes robustness (i.e., sub-linear dependence of the signal to the protein concentration), this robustness is maintained if an oncogene activates the pathway above the feedback, while robustness disappears when the oncogene acts within the feedback loop. **d** In colon cancer cells, signaling is robust due to a feedback to CRAF. **e** MEK inhibition is less effective due to negative feedback. When the feedback is intact, a high concentration of the inhibitor is required to reduce the signal significantly. If cells harbor a BRAF-V600E mutation, the feedback is disrupted and consequently lower amounts of inhibitor can reduce the signal strongly

layers of intertwined feedback, it is generally difficult to assess which of these feedbacks is responsible solely by measuring the feedback phosphorylation sites. We thus thought of a different strategy: to use oncogenes to dissect the network. These oncogenes are not viewed as perturbations, but used to activate the pathway at different points in the network, and subsequently study robustness. The reasoning was the following: if an oncogene activates the pathway downstream of the point, where the signal feeds back, the action of the feedback is disrupted. Thus, if signaling robustness is due to a feedback upstream, cells harboring this oncogene should show no robustness. In contrast, if the oncogene activates the pathway upstream, the feedback is intact and the pathway should retain its robustness (Fig. 3c). By using a panel of colon cancer cell lines, we found that cells harboring an oncogenic mutation in KRAS show robustness, while cells showing a V600E mutation in BRAF, which is just downstream of KRAS, are no longer robust, thus they show linear dependence of phospho-ERK on total ERK levels. This suggested that a feedback on RAF, possibly CRAF, was the main feedback causing robustness, which we confirmed by further mechanistic studies. For example, in HEK cells ERK level was robust against perturbation. If a chemically inducible CRAF without regulatory domain was activated, the pathway loses robustness, suggesting that the pathway is only robust when the feedback to the regulatory domain of CRAF is present. Measurement of CRAF hyper-phosphorylation confirmed that RAF was strongly differentially phosphorylated depending on MEK activity, and a RAS activity assay confirmed that RAS activity remains constant when MEK is perturbed.

Negative feedback needs to be rather strong in order to cause notable signaling robustness, and we found that the logarithmic gain in the feedback is about two times as strong as the logarithmic gain within the signaling pathway itself. Interestingly, other studies that aimed at quantifying feedback loops in EGFR signaling also attributed the strongest feedback to the feedback from ERK to RAF [65], possibly mainly CRAF in colon cells (Fig. 2d). This raises

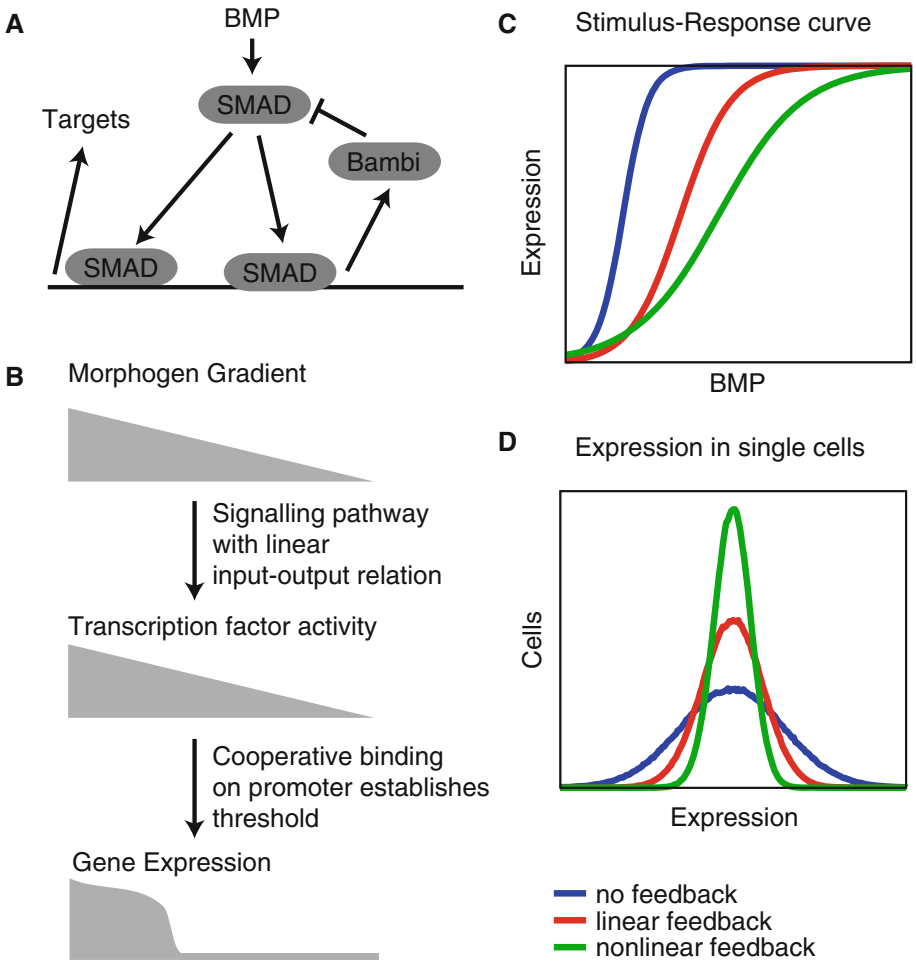

Fig. 3 Robustness and dose–response linearization in BMP signaling. **a** Schematic representation of the BMP signaling cascade. BMP binding to cognate cell-surface receptors activates SMAD transcription factors which induce target gene and BAMBI expression. **b** Processing of the BMP signal in living embryos. The extracellular BMP gradient results in a gradual activation of SMAD transcription factors along the morphogenetic field. Sharp boundaries are observed at the level of target gene expression, possibly by cooperative binding of transcription factors to target promoters. **c** Dose–response linearization by negative feedback. The steady-state expression of a BMP target gene is plotted as a function of the BMP concentration (according to a simple model described in [59]). Increasingly nonlinear feedback (*green, red*) linearizes the dose–response curve when compared to a corresponding feedback-less system (*blue*). Nonlinearity was introduced by assuming that BAMBI inhibits SMAD activity with an exponent larger than 1. **d** Negative feedback suppresses cell-to-cell variability in target gene expression. Histograms of target gene expression were calculated based on 1,000 simulations (each simulation representing one cell). Correlated fluctuations in target gene and BAMBI expression were introduced by sampling the corresponding synthesis rates from the same log-normal distribution to represent extrinsic gene expression noise in the model

the question about the role of the other feedback in the system, which is still open. Possibly these feedback loops are operating in different signaling regimes. Similarly, it has been observed the two transcriptional feeback loops in STAT signaling operate at different regimes, one at high- and the other at low-level stimulation [66].

Several groups were interested in the consequences of such strong feedback for the efficiency of targeted inhibitors. Theoretical analysis has predicted that this strong feedback would make the signaling pathway robust against pharmacological intervention, and this prediction was confirmed recently by quantitative experimentation [44]. Specifically, MEK inhibition by small molecule inhibitors is impaired, since inhibition of MEK results in reduced feedback, thus MEK inhibition can be partially compensated by higher signal (Fig. 2e). From our study, we learned that BRAF V600E mutations lead to a breakdown of robustness, suggesting that BRAF mutation would lead to a better efficiency of MEK inhibitors [44, 58, 67]. Interestingly, in clinical trials in melanoma patients, where BRAF V600E mutations are common, response to the MEK inhibitor Selumetinib seems better when BRAF is mutated [68]. Thus, the feedback structure in tumor cells may be important to predict good drug targets. Robustness

due to feedback is on the one hand important for proper functioning of signal transduction, on the other hand, it makes it difficult to intervene with signaling. Interestingly, broken feedback due to oncogenes may result in differential robustness: cells that show impaired feedback (the diseased cells) can be drugged, while the healthy cells with intact feedback are not strongly affected.

Robustness in BMP signaling

A central step during embryonic development is the spatial patterning of specialized cell types within a tissue. Patterning is determined by so-called morphogen-gradients. A morphogen is an extracellular diffusible factor that spreads from a localized source and forms a concentration gradient across a developing tissue. How can a single extracellular gradient establish a spatially ordered array of multiple cell types? According to the so-called "French-flag model", the target cells acquire different fates depending on the morphogen concentration they experience. Thus, multiple decision thresholds exist in each cell, allowing the tissue to read the gradient.

The bone morphogenetic proteins (BMPs) play a central role in early embryonic patterning in *Drosophila* and in vertebrates. In *Drosophila*, the BMP family member Decapentaplegic (Dpp) acts as a morphogen that establishes the correct patterning of imaginal discs [69]. In *Xenopus laevis*, the BMP4 gradient establishes dorso-ventral patterning of the gastrula marginal zone [70]. BMPs elicit cellular responses by binding to their cognate BMP receptors which in turn phosphorylate and thereby activate SMAD1/5/8 transcription factors. SMAD1/5/8 hetero-trimerize with the SMAD4 co-factor upon phosphorylation, and the whole complex translocates into the nucleus, where it induces target gene expression (Fig. 3a). The BMP signaling pathway is characterized by several transcriptional negative feedback loops, one of which is shown in Fig. 3a: activated SMAD transcription factors induce the expression of the receptor inhibitor BAMBI (BMP and activin membrane-bound inhibitor homolog), thereby shutting off signal transduction.

Several experiment–theory studies analyzed the in vivo robustness of BMP morphogen and SMAD phosphorylation gradients towards intrinsic and extrinsic fluctuations (reviewed in [16, 22]). The BMP gradient was shown to be robust against variations in the expression of the proteins involved in gradient formation [71]. Moreover, BMP gradients were shown to scale with embryo size, thus allowing the system to maintain the proportions of patterning despite variations in embryo size [22, 72, 73]. These analyses concluded that robustness requires intercellular communication mechanisms. For example, gradient robustness relies on bifunctional extracellular proteins (e.g., Sog), which inhibit BMP activity and at the same time enhance BMP diffusion in the morphogenetic field [71].

Whether cell-autonomous BMP signaling mechanisms also promote robustness is less well established. In *Xenopus* embryos, the BMP4 gradient induces at least four different cell types, and cell fate specification arises at the level of SMAD target gene expression [70]. In the simplest model, cell fate specification and patterning are initiated by cooperative binding of SMAD factors to target gene groups with different affinities, thus converting the BMP/SMAD activity gradient into sharp spatial boundaries (Fig. 3b). Based on this model, the signaling pathway should transduce the incoming BMP signal in a linear manner (Fig. 3b). In fact, single-cell measurements reveal that increasing BMP4 doses induce a gradual increase in the SMAD1/5/8 in vitro and in the living embryo [59, 71]. Moreover, the affinity-driven cell fate specification mechanism can only sense the absolute phospho-SMAD signal in the nucleus, suggesting that variations in signaling protein expression need to be compensated. One candidate robustness mechanism is negative feedback, which has been reported to linearize biochemical dose–response curves, while reducing variability [45, 74]. In a recent study, we indeed found that cell-autonomous negative feedback regulation via BAMBI allows for a gradual BMP response over a broad concentration range, and at the same time confers protein concentration insensitivity [59].

A simple mathematical model suggested that BAMBI-mediated feedback should affect the stimulus–response curve of the system in two ways: negative feedback shifts the half-maximal stimulus to the right, thus desensitizing signal transduction (Fig. 3c). Moreover, negative feedback regulation reduces the steepness of the stimulus–response curve and thereby expands the range of BMP concentrations that can be sensed by the signaling cascade (Fig. 3c). We experimentally confirmed these effects by measuring stimulus–response curves of the BMP signaling cascade. Pathway activity was monitored by immunoblotting against phospho-SMAD proteins and by assessing the expression of reporter genes harboring BMP-responsive elements. Perturbation of BAMBI-mediated feedback by knockdown or constitutive overexpression of BAMBI resulted in a steeper dose–response curve when compared to control cells [59]. Thus, transcriptional feedback via BAMBI ensures that the SMAD signaling cascade transduces the BMP signals in a linear manner.

Eukaryotic signaling cascades typically exhibit strong cell-to-cell variability. Fluctuations are thought to mainly arise from variability in initial signaling protein expression levels [11]. This effect, together with extrinsic noise in gene expression, establishes strong correlated fluctuations in target gene expression [75]. Theoretical studies

suggested that transcriptional negative feedback loops might exploit these correlated fluctuations to reduce target gene expression variability [57]: if a cell expresses high levels of a target gene, it will also express high levels of the feedback inhibitor, thereby lowering signal transduction and correcting for target gene fluctuations. Interestingly, such a mechanism could ensure that a single transcriptional feedback regulator simultaneously reduces the variability of many target genes. Figure 3d shows cell-to-cell variability simulations of a simple mathematical model, and demonstrates the robustness-promoting role of negative feedback. The model predictions were confirmed experimentally by analyzing the expression GFP-reporter under control of a BMP-responsive element at the single-cell level. As expected, the depletion of the feedback inhibitor BAMBI by siRNA-mediated knockdown broadened the distribution profile of target gene expression in cultured cells. This effect appeared to be specific to negative feedback regulation, as the depletion of non-feedback inhibitors of BMP signaling had no effect on the variability of target gene expression [59]. The expression of BMP target genes was also analyzed in developing embryos to confirm the robustness-promoting role of negative feedback in vivo. As expected, BMP target gene expression in living embryos was more variable when BAMBI was depleted by antisense-mediated knockdown. Consistent with a role of BMP target genes in embryonic patterning, BAMBI depletion further increased the variability of morphological features of living embryos (e.g., tail length and eye size) [59]. This supports that BAMBI feedback promotes robustness under physiological conditions.

Many growth factor signaling cascades including the BMP pathway are organized in so-called synexpression groups [54, 76]. This means that positive and negative regulators of the pathway as well as target genes are coordinately induced in response to stimulation. Why signaling pathways are organized in synexpression groups is not clear. From a mathematical point of view, the simultaneous induction of multiple negative feedback regulators introduces nonlinearity in feedback regulation if they act at different steps of the signaling cascade ("multistep ultrasensitivity"). The simulations in Figs. 3c, d show that the feedback effects are generally more pronounced if transcriptional feedback regulation is assumed to affect SMAD transcription factors with an exponent larger than one ("nonlinear feedback"). Functional organization of BMP signaling in a synexpression group may thus optimize feedback performance with respect to dose–response linearization and variability suppression. Accordingly, experimental studies confirm that the variability in BMP target gene expression increases strongly upon simultaneous depletion of multiple negative feedback regulators in the pathway [59]. A question that remains to be addressed by modeling is why synexpression groups frequently encompass positive feedback loops as well. We speculate that a combination of positive and negative feedback loops may establish correlated fluctuations in the levels of antagonistic signaling proteins, thereby suppressing cell-to-cell variability [77].

Taken together, intracellular transcriptional feedback and intercellular communication mechanisms appear to cooperate in promoting robustness of BMP-induced spatial patterning.

Conclusions

While most studies on robustness have been conducted in bacteria, recently several groups have started to investigate robustness in higher organisms. These studies have either focused on development, where it is clear that signaling has to robustly transmit information about the quantity of a morphogen, or signaling in cancer, where robustness of signaling pathways hampers the action of drugs. Strikingly, in both cases negative feedback plays a pivotal role in generating robust behavior of the signaling pathway. This is in contrast to several bacterial systems, where the motif of incoherent feed-forward loops (either due to co-expression of proteins, or due to bifunctionality of enzymes) provides robustness. It is too early to say whether this difference is solely due to the investigated signaling systems, or it there is a general difference in design of signaling pathways in bacteria and higher eukaryotes. Other mechanisms that have theoretically been predicted to mediate robustness [63] or simply redundancy are currently underexplored. Current mathematical models are often unable to fully capture the robustness of biological systems, especially if strong perturbations are considered. For example, a recent experimental study revealed that a quantitative cell cycle model failed to predict how strongly the expression of network components can be up or downregulated without a disruption of network functionality [78]. Most likely, this model neglected important compensation mechanisms such as functional redundancies, long-term transcriptional regulation and mutual stabilization of network components. Another underexplored aspect of robustness is how the temporal dynamics of signal transduction can be made invariant. Most current computational models show robust steady state signal transduction, while the temporal dynamics are highly parameter-sensitive [31]. While such temporal variability may be tolerable in some signaling pathways, it may be deleterious if the pathway dynamics determine cellular decision making. We believe that the emerging fields of quantitative experimentation and mathematical modeling will shed more light on how robustness in signaling is implemented in near future. Also, novel

theoretical approaches from information theory and engineering may help to uncover how robust these pathways are, and which mechanisms confer robustness to signaling pathway.

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