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Strong negative feedback from Erk to Raf confers robustness to MAPK signalling

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Protein levels within signal transduction pathways vary strongly from cell to cell. Here, we analysed how signalling pathways can still process information quantitatively despite strong heterogeneity in protein levels. We systematically perturbed the protein levels of Erk, the terminal kinase in the MAPK signalling pathway in a panel of human cell lines. We found that the steady-state phosphorylation of Erk is very robust against perturbations of Erk protein level. Although a multitude of mechanisms exist that may provide robustness against fluctuating protein levels, we found that one single feedback from Erk to Raf-1 accounts for the observed robustness. Surprisingly, robustness is provided through a fast post-translational mechanism although variation of Erk levels occurs on a timescale of days.
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Introduction

Mammalian cells harbour a complex and highly interlinked signal transduction network that relays information from the outside of the cell to the inside, thereby controlling different processes such as cytoskeletal reorganisation, translation and the action of transcription factors. One of the central challenges in understanding how cells process information in order to respond adequately to their environment is to understand how the signalling network deals with uncertainty and the noise inherent to biochemical processes. The role of noise in signalling networks due to stochasticity of the biochemical reactions has been addressed in detail during the last decade (Elf and Ehrenberg, 2003; Bhalla, 2004; Hornung and Barkai, 2008; Bruggeman *et al*, 2009). Signal transduction is often realised by reversible post-translational modifications. Although the noise level due to these post-translational modification events can be relatively high, the timescale of fluctuations is usually considered too small to be transmitted to gene expression (Bruggeman *et al*, 2009) and will therefore hardly influence major cellular decisions.

However, increasing evidence suggests that the most important source of uncertainty in mammalian signal transduction is noise in the expression of signalling proteins. Even clonal populations display strong cell-to-cell variations of the level of the same protein with a s.d. of 20–30% of the mean (Sigal *et al*, 2006). These fluctuations of protein concentrations have been shown to influence major cellular decisions, such as apoptosis (Spencer *et al*, 2009). For Erk2, the terminal kinase of the mitogen-activated signal transduction (MAPK) pathway, strong variations of the protein level have been found when single cells are compared with each other. Cells within the lowest and highest 10% percentile exhibit a three-fold difference in Erk2 levels, respectively (Cohen-Saidon *et al*, 2009). In light of these observations, it appears surprising that a biochemical machinery exhibiting large differences and fluctuations of the levels of its components is nevertheless capable of processing information reliably. One would rather expect that cells might respond very heterogeneously to the same stimulus, depending on the intracellular levels. Yet, the MAPK pathway is centrally involved in cellular decision-making, and small quantitative differences in pathway activity can result in large changes in cellular phenotype (Ghiglione *et al*, 1999; Santos *et al*, 2007; Blüthgen and Legewie, 2008; Nakakuki *et al*, 2010). Importantly, Erk activity is also a very sensitive gate-keeper in the G1/S transition

(Yamamoto *et al*, 2006) and the quantity of Erk activity is directly linked to cell growth (Lefloch *et al*, 2009). Despite the observed variations in Erk levels, imprecise regulation of the cell cycle and growth seems implausible. This suggests that the signalling network is equipped with mechanisms that render the biological pathway readouts robust against variations of its components.

For various signal transduction networks, different design principles have been identified that confer robustness to the network against typical perturbations of its constituents (for review, see Stelling *et al*, 2004). For example, it has been shown that the *Drosophila* segment polarity network is very robust against fluctuations in the kinetic properties of its components (von Dassow *et al*, 2000). Negative feedback is a very frequent motif found to provide robustness, for example, in gene regulatory networks (Becskei and Serrano, 2000), in baker's yeast galactose network (Acar *et al*, 2010) and in bacterial chemotaxis (Alon *et al*, 1999). While these examples show how developmental pathways, simple eukaryotes and bacteria deal with uncertainty, little is currently known about how robustness is realised in the mammalian signal transduction network. It is of central importance to identify which principles govern robustness in mammalian signalling pathways, since perturbation of these pathways by pharmacological agents nowadays is a main strategy in fighting diseases including cancer (Zhang *et al*, 2009). Since many unknown obstacles can prevent efficient inhibition of signalling pathways in cancer, perturbation strategies need to be identified that successfully manipulate the signalling network and at the same time are not hampered by the natural robustness of the system. Moreover, identification of reduced robustness, due to oncogenic mutations in cancer cells, will increase the chance of successful targeted intervention.

We, therefore, set out to investigate how robustness to protein expression is realised in one of the best-studied mammalian signal transduction system, the MAPK signal transduction pathway. We chose a combined experimental and theoretical approach to determine how a change in total protein concentration of the terminal kinase in this pathway, Erk, translates into changes of its phosphorylated active form, and which consequences arise for signalling and pathway intervention upstream of Erk.

Results

Mathematical analysis predicts linear relation between protein level and activity

The activity of the terminal kinase of the classical MAPK signal transduction pathway, Erk, is controlled by competition of phosphorylation and dephosphorylation of a threonine/tyrosine motive. Phosphorylation is carried out by the kinase Mek, and Erk is dephosphorylated by a multitude of phosphatases. The biochemical processes involved in Erk phosphorylation have been elucidated in depth. It has been shown that phosphorylation by Mek proceeds sequentially, tyrosine being phosphorylated before threonine (Schilling *et al*, 2009). Additionally, Mek tends to detach from Erk before carrying out the second phosphorylation (Ferrell and Bhatt, 1997), that is, phosphorylation is not processive. Dephosphorylation is less well studied, but it is likely that it follows a similar scheme. Furthermore, it has been demonstrated that both isoforms of Erk, Erk1 and Erk2, are nearly identical in their biochemical properties (Yoon and Seger, 2006; Lefloch *et al*, 2008; Voisin *et al*, 2010). From this information, we developed a simple mathematical description of Erk activation where the steady-state level of double-phosphorylated Erk (ppErk) is dependent on the phosphorylation rate (k_m), the dephosphorylation rate (k_p) and the total level of Erk (Erk_T) in the following form (derivation see Supplementary information):

$$\mathrm{ppErk} = \mathrm{Erk_T} \times \frac{\left(\frac{k_m}{k_p}\right)^2}{1 + \left(\frac{k_m}{k_p}\right) + \left(\frac{k_m}{k_p}\right)^2}. \quad (1)$$

This equation shows that the double-phosphorylated form of Erk (ppErk) is predicted to be non-linearly dependent on the phosphorylation and dephosphorylation rates. The exact form of the second term on the right hand side is being determined by the details of the kinetic scheme, such as the number of phosphorylation sites, or whether there is cooperativity between phosphorylation of the different sites. However, the model predicts a linear dependence of ppErk on protein concentration Erk_T, independent of the precise kinetic mechanism as long as the kinase Mek and the phosphatases are not strongly saturated. Consequently, 30% variation in Erk levels, for example, due to gene expression noise, would translate into 30% variation in phosphorylation of Erk in a population of clonal cells (see Figure 1A and B, green lines).

In order to quantify how ppErk depends on Erk_T, it is instrumental to use the normalised derivative, R:

$$R = \frac{\mathrm{Erk_T}}{\mathrm{ppErk}} \frac{\mathrm{d\,ppErk}}{\mathrm{d\,Erk_T}}. \quad (2)$$

This coefficient is very similar to response coefficients from modular response analysis, which defines information transfer in systems where no mass-flow exists between variables (Kholodenko *et al*, 2002). Since the mathematical properties and, most importantly, the interpretation of the above defined coefficient are very similar, we will refer to it as response coefficient. For our model, it equates to one, since the phosphorylated form of the protein changes proportionally with the total protein concentration. In this case, the pathway is non-robust. Values below 1 denote increased robustness of the phosphorylated form, and R=0 indicates perfect insensitivity of ppErk against changes in Erk levels.

Examples of theoretical ppErk levels due to variations in total Erk concentration for a hypothetical non-robust, a partially robust and fully robust system are depicted in Figure 1A. Taking a realistic distribution of total Erk concentrations in single cells (log-normal with s.d. of 20% of mean), a non-robust system would show strong variations in ppErk levels (Figure 1B), with some cells showing very low levels and other levels more than two-fold higher than the mean. In contrast, a system with increased robustness (e.g., R=1/3, red lines in Figure 1A and B) would show a strongly reduced spread of ppErk levels. A fully robust system (R=0, blue lines in Figure 1A and B) would virtually eliminate variations in ppErk levels between clonal cells.

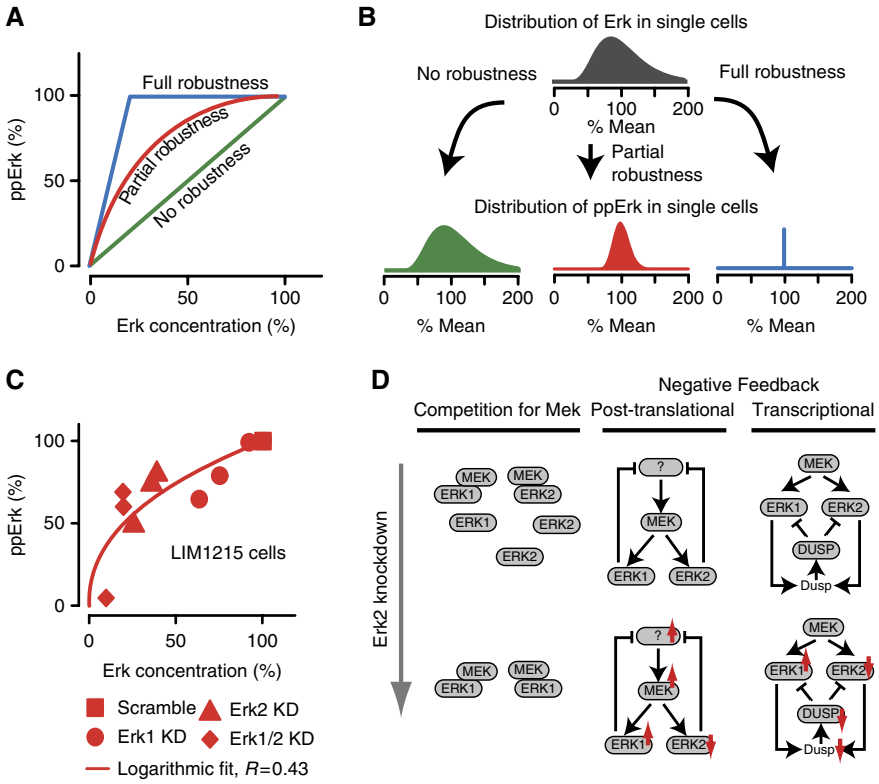

Figure 1 Robustness of MAPK signalling. (**A**) Mathematical analysis of Erk phosphorylation kinetics suggests that the phospho-Erk level depends linearly on Erk protein concentration (green line, no robustness). The red line shows hypothetical partial robustness, where phosho-Erk level depends sublinearly on Erk. The blue line corresponds to a fully robust system, where phosho-Erk can fully compensate loss of Erk. (**B**) The consequences of variability in Erk expression (grey) on phospo-Erk expression for a non-robust, partially robust and fully robust system are shown. (**C**) Steady-state phospho-Erk level of LIM1215 cells depends only weakly on Erk concentration. Each dot shows quantified pan-isoform phospho-Erk and Erk levels from western blots of cells treated with siRNA against Erk1 or Erk2 alone, Erk1 and Erk2 in combination in percent of the scrambled control. (**D**) Possible mechanisms providing robustness illustrated for knockdown of Erk2: competition for upstream kinase Mek, where loss of Erk2 results in higher access of Erk1 to Mek; post-translational negative feedback, where loss of Erk2 results in relieve of negative feedback and therefore stronger upstream signalling; and transcriptional negative feedback, where knockdown of Erk2 results in decreased concentrations of deactivating phosphatases. Source data is available for this figure at www.nature.com/msb.

Phospho-Erk levels depend only weakly on total Erk levels

In order to test whether phosphorylated Erk is linearly dependent on the level of the protein, as our initial model suggests, we took advantage of the fact that cells express two isoforms of Erk, Erk1 and Erk2. These isoforms are expressed at different levels, with Erk2 being the dominantly expressed isoform (Lefloch *et al*, 2008; Voisin *et al*, 2010). By siRNA-mediated knockdown of each isoform alone and both isoforms together we could perturb the total level of Erk to different extends. We performed such knockdown experiments in LIM1215 cells, colorectal cancer cells without mutations upstream or within the MAPK signalling pathway (Whitehead *et al*, 1985; Jhawer *et al*, 2008; and data not shown). Subsequently, we quantified the levels of Erk and ppErk using quantitative western blotting (details see Materials and methods).

Figure 1C summarises the results of this experiment. We expected from the mathematical model that Erk phosphorylation is proportional to the total Erk concentration. However, our western blot analyses clearly show that ppErk levels deviate strongly from the model prediction. The data rather suggest that even a reduction of Erk levels by 80% will only lead to a modest decrease of phosphorylated Erk down to 50%, thus indicating that ppErk is rather robust against variations in Erk levels. By linear regression of the logarithmic values we estimate R=0.43 (fit shown as solid line). A representative blot is shown in Supplementary Figure S1.

Three possible mechanisms for robustness

Surprised by the high robustness of phosphorylated Erk levels towards strong variations in total Erk levels, we asked which mechanisms may lead to this remarkably low sensitivity of Erk phosphorylation. We speculated that three properties of this signalling pathway may lead to such low sensitivity and analysed the consequences of these potential mechanisms further utilising mathematical models. These properties are as follows:

Saturated phosphorylation by Mek

It is known that Mek is only phosphorylated at low levels (Lefloch *et al*, 2008), and that the docking interaction between Mek and Erk can be tight (Kolch, 2000). Therefore, Mek might

be saturated by Erk, that is, most phosphorylated Mek is bound to Erk. In that case, lowering the total Erk concentration would make Mek more accessible to the remaining Erk molecules and increase the probability of their phosphorylation (depicted in Figure 1D, left panel). In the following, we show that already a simple model that assumes enzymatic activation of Erk by a single phosphorylation event entails robustness of the level of phosphorylation or a response coefficient smaller than 1, respectively. The model reads:

$$\mathrm{pErk}\cdot k_\mathrm{p} = \frac{\upsilon_\mathrm{m}\cdot(\mathrm{Erk_T}-\mathrm{pErk})}{K_\mathrm{M}+\mathrm{Erk_T}-\mathrm{pErk}}. \quad (3)$$

The associated response coefficient reads:

$$R = \frac{\mathrm{Erk_T}\cdot K_\mathrm{M}\cdot\left(1-\frac{\mathrm{dpErk}}{\mathrm{dErk_T}}\right)}{(\mathrm{Erk_T}-\mathrm{pErk})(K_\mathrm{M}+\mathrm{Erk_T}-\mathrm{pErk})}. \quad (4)$$

Since $\frac{\mathrm{dpErk}}{\mathrm{dErk_T}}$ ranges between 0 and 1 (see Supplementary information), and typically only a small fraction of Erk is phosphorylated in steady state ($\mathrm{pErk} \ll \mathrm{Erk_T}$), R simplifies to:

$$R \approx \frac{\mathrm{Erk_T}\cdot K_\mathrm{M}\cdot\left(1-\frac{\mathrm{dpErk}}{\mathrm{dErk_T}}\right)}{\mathrm{Erk_T}\cdot K_\mathrm{M}+\mathrm{Erk_T^2}} < 1. \quad (5)$$

The prediction of the model thus is that the pathway is robust if pMek levels are low and unaffected by a change in Erk levels. Furthermore, increasing concentrations of Mek will reduce saturation of Mek, and would therefore increase ppErk levels.

Post-translational feedback regulation

The MAPK signalling pathway is regulated by post-translational feedback at many different levels. Erk has been shown to phosphorylate and thereby inactivate several adaptor molecules, and Erk deactivates Raf-1 by phosphorylating inhibitory sites (Dougherty *et al*, 2005; Yoon and Seger, 2006; Dhillon *et al*, 2007). The consequence of such negative feedback when reducing the concentration of one of the isoforms is illustrated in Figure 1D. Since the amount of ppErk is reduced, feedback inhibition is relaxed, and consequently the level of pMek increases. This in turn increases the phosphorylation of the remaining Erk isoform and the residual protein of the targeted isoform, thereby partially compensating for the loss of protein.

A mathematical analysis of the feedback system yields that the response coefficient R can be expressed in terms of the feedback strength. If one adds a negative feedback to the simplest model of Equation (1), the steady state of the system can be described by two variables, pMek and ppErk:

$$\begin{aligned}\mathrm{ppErk} &= f(\mathrm{Erk_T},\ \mathrm{pMek})\\ \mathrm{pMek} &= g(\mathrm{ppErk}).\end{aligned} \quad (6)$$

Calculating R from these equations yields:

$$R = \frac{1}{1-r_1 r_2}, \quad (7)$$

with the local response coefficient $r_1 = (\partial \ln \mathrm{pMek})/(\partial \ln \mathrm{ppErk})$ quantifying the strength of the feedback, and $r_2 = (\partial \ln \mathrm{ppErk})/(\partial \ln \mathrm{pMek})$ quantifying the amplification from pMek to ppErk. Since the feedback and consequently r_1 is negative, an increased strength of the negative feedback reduces R. Therefore, negative feedback may confer robustness to ppErk levels.

If negative feedback is the cause of robustness then we would expect that, $|r_1\ r_2| > 1$, since we previously estimated the global response coefficient R to be 0.43. Therefore, we would predict that a feedback needs to be strong, that is, changes in ppErk are amplified along the feedback loop.

Transcriptional feedback

Erk is not only regulated via feedbacks at the post-translational level, but also by transcriptional negative feedback loops. So-called dual-specificity phosphatases (DUSPs) constitute a protein family, which can dephosphorylate threonine and tyrosine residues. A subfamily of these DUSPs is able to bind to Erk and is involved in Erk dephosphorylation, mainly DUSPs 2, 4, 5, 6, 7 and 9 (Patterson *et al*, 2009). The expression of several of these DUSPs is controlled by transcription factors downstream of Erk thereby constituting negative feedback regulation (Amit *et al*, 2007; Legewie *et al*, 2008). Negative transcriptional feedback may provide robustness similar to post-translational feedback. If Erk concentration is reduced, expression of the inhibitory protein will be lowered and in turn the remaining Erk will be hyper-phosphorylated. Analogously to the post-translational feedback, also the transcriptional feedback needs to be strongly amplifying.

Since each of the three mechanisms may help in compensating the changes in total Erk levels, we set out to investigate which of them, either alone or in combination, does confer robustness to Erk phosphorylation.

Transcriptional negative feedback regulation via DUSPs is not involved in robustness of Erk phosphorylation

We first investigated whether the observed robustness of ppErk, on a timescale of days, can be explained by transcriptional negative feedback. In order to test whether the observed robustness can be attributed to feedback regulation by DUSPs, we analysed Erk-mediated regulation of DUSPs on the transcriptional level in LIM1215 cells. We applied the Mek inhibitor U0126 to the cells, and observed that the phosphatases DUSP5, DUSP6 and DUSP7 are downregulated, thereby constituting a negative feedback (Figure 2A).

Since we identified three DUSPs as feedback regulators of Erk, we performed quantitative RT–PCR analyses of these DUSPs together with seven other DUSP genes after knockdown of both Erk isoforms. The results, as depicted in Figure 2B, show that none of the classical DUSPs displayed a strong downregulation 48 h following Erk knockdown. Therefore, although transcriptional negative feedback regulation via DUSPs is present in these cells, such feedback via DUSPs can be ruled out as a mechanism for the observed robustness in Erk phosphorylation.

Post-translational feedback via Raf-1 mediates robustness

As the next hypothesis, we asked whether post-translational feedback is involved in compensating the reduction of Erk levels. To investigate this, we took advantage of a panel of colon cancer cells that harbour different mutations upstream

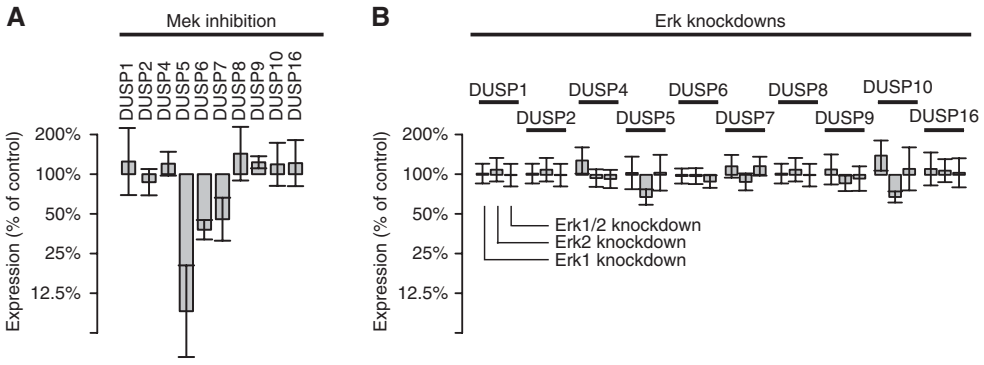

Figure 2 Transcriptional negative feedback is present but not utilised to compensate variations in Erk protein level. The bar graphs show the expression of several classical dual-specificity phosphatases (DUSPs) measured using q-RT–PCR in comparison with the level in cells treated with control (DMSO or scrambled, in (A) or (B), respectively). (**A**) DUSP5, 6, 7 are negative feedback regulators in LIM1215 cells, as treatment with high doses of the Mek inhibitor U0126 resulted in strong downregulation of DUSP5, 6, 7. (**B**) None of the DUSPs shows significant downregulation after knockdown of Erk1 and/or Erk2 in LIM1215 cells. Source data is available for this figure at www.nature.com/msb.

of Erk. These mutations constitutively activate the signal transduction pathway at different levels. Whereas the cell lines HT29 and RKO have an activating mutation in B-Raf (V600E), the cell lines HCT116 and SW480 contain mutations in Exon 2 of the *K-Ras* gene (D13E and G12V, respectively). As control, the LIM1215 cell line is used that is devoid of KRAS or BRAF mutations. The left panel of Figure 3A shows the position of these proteins in the MAPK signalling pathway. K-Ras activates the pathway primarily via Raf-1, whereas B-Raf activates Mek and signals independently of Raf-1 (Karreth *et al*, 2009).

In consequence, we expected that if a post-translational feedback acts upstream of Ras, only LIM1215 cells, which are devoid of mutations in the pathway, should show robustness in ppErk levels. If the feedback acts at Raf-1, the expectation is that Ras-mutated cells still show robustness, but B-Raf-mutated cells would not.

We performed knockdown experiments of the two isoforms of Erk in these cells and measured the total Erk level as well as the levels of phosphorylated Erk1 and Erk2. As a first result, this analysis showed that there is no compensatory regulation between Erk1 and Erk2 proteins, that is, if Erk1 protein level is lowered, this is not compensated by Erk2 protein expression and vice versa, independently of the cellular background (see Figure 3B).

We then calculated the total amount of Erk and phospho-Erk after knockdown in this panel of cell lines. Again, we then analysed whether ppErk depends non-linearly on Erk concentration. Strikingly, we find that in both cell lines with a B-Raf V600E mutation, robustness is lost, that is, the phosphorylation of Erk decreases linearly with removal of Erk (Figure 3C, blue data points). In stark contrast, cells harbouring a mutation in Ras show a non-linear relation between ppErk and Erk (Figure 3C, red data points). By regression of the logarithmic values, we estimate the global response coefficient to be R=0.36 and R=0.20 for HCT116 and SW480, respectively. The western blots (Figure 3D; Supplementary Figure S1) show consistently a strong increase in Erk1 phosphorylation if Erk2 is knocked down, for cells with wild-type B-Raf, and unchanged Erk1 phosphorylation if B-Raf is mutated (see Supplementary Figure S2). These data suggest that there is an absence of robustness in B-Raf-mutated cells, whereas Ras-mutated cells or cells harbouring wild-type Raf and Ras show robust compensation of loss in Erk expression. We hypothesise that this is due to a negative post-translational feedback targeting Raf-1, as mutated B-Raf triggers MAPK activity independently of Raf-1.

In order to further investigate the hypothesis that robustness is caused by a feedback to Raf-1, we measured pMek levels in our cell line panel after knockdown of Erk isoforms. If feedback regulation is indeed mediated by Raf-1 or another component between Ras and Mek, one would expect an increase of pMek levels after knockdown of the Erk isoforms in Ras-mutated cells. In contrast, one would expect no increase of pMek in B-Raf-mutated cells. As expected, pMek levels increase in Ras-mutated cells after knockdown of Erk1, Erk2 or both isoforms in Ras-mutated cells (Figure 3E, red bars). The increase in pMek level is low following Erk1 suppression, higher after Erk2 suppression and highest following Erk1/2 double knockdown. This indicates that the effect of the feedback increases monotonically with decreased Erk protein abundance. In contrast, no increase in pMek after Erk knockdown was observed in B-Raf-mutated cells (Figure 3E, blue bars).

In Ras-mutated cells, Raf-1 is feedback controlled whereas Ras is not

In order to confirm that Raf-1 is feedback controlled in colon cancer cell lines, we applied the Mek inhibitor U0126 and its vehicle control DMSO for 24 h to HT29 and HCT116 cells to abrogate Erk activity, and monitored the Raf-1 phosphorylation status using antibody recognising the cluster of Erk phosphorylation sites within the hinge region of Raf-1 (Dougherty *et al*, 2005). In agreement with the chronic activation of the Erk pathway in these colon cell lines, Raf-1 displays a prominent phosphorylation at these residues in the control samples, but not in U0126-treated cells (Figure 4A, top row). Furthermore, the detection of total Raf-1 demonstrated that the application of U0126 accelerated its electrophoretic mobility (Figure 4A, middle row), which is in full agreement with various previous studies using the chemically distinct MEK inhibitor PD98059 (Wartmann *et al*, 1997; Dougherty *et al*, 2005). Interestingly, Raf-1 is also feedback controlled

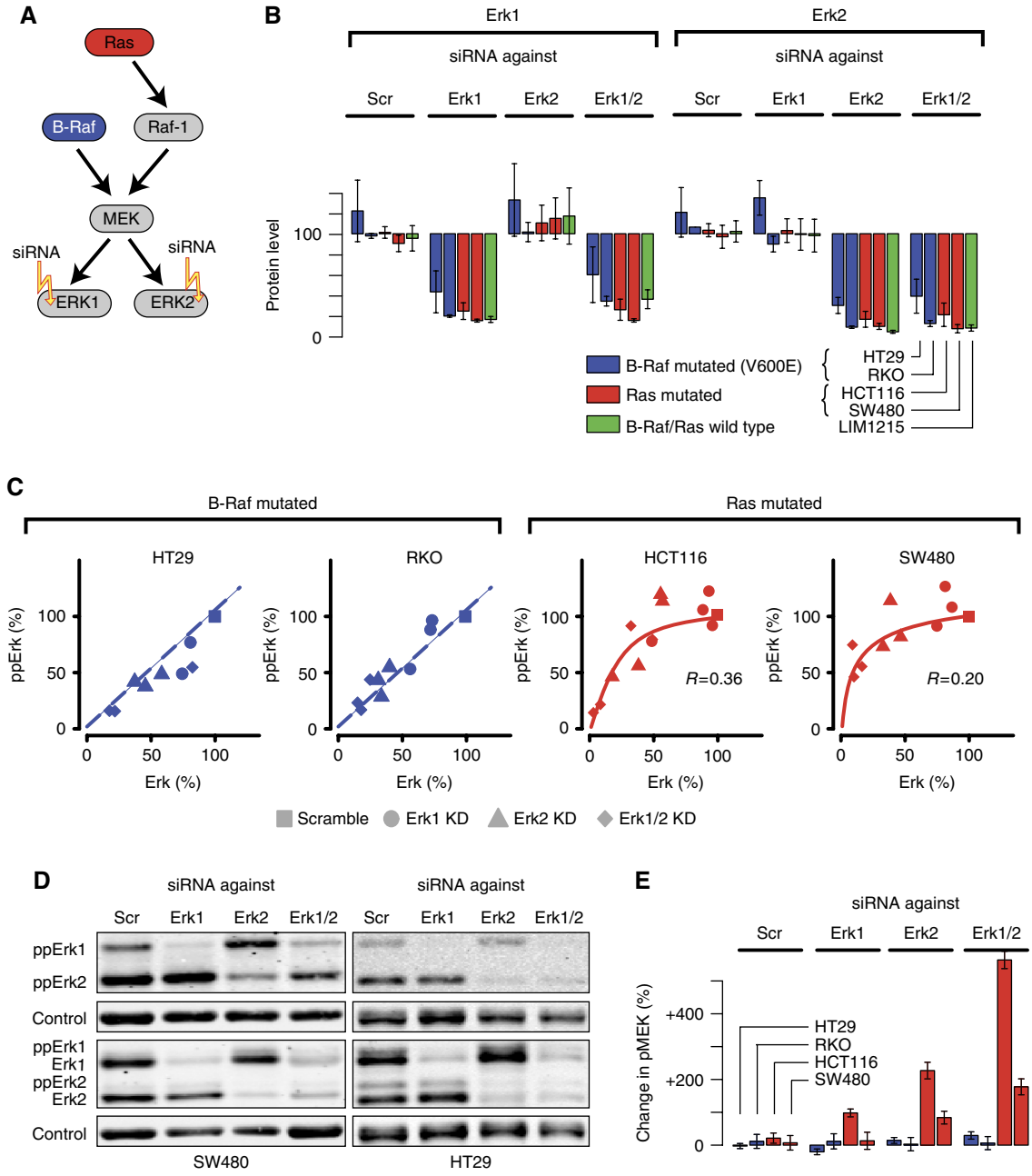

Figure 3 Detailed analysis of post-translational compensation of varying Erk concentration. (**A**) Position of mutations of the analysed cells in the pathway: five colon carcinoma cell lines were analysed, LIM1215 has no mutation in the MAPK signalling pathway, HT29 and RKO express constitutively active B-Raf (V600E), and SW480 and HCT116 harbour an activating mutation in K-Ras. (**B**) Changes in expression of Erk1 (left) and Erk2 (right) 48 h after treating the cells with scrambled control siRNA or siRNA targeting Erk1, Erk2 or both isoforms in the five cell lines. If one isoform is knocked down, no significant change in the other isoform can be observed. (**C**) Pan-isoform Erk and phospho-Erk levels after knockdown of Erk1 and/or Erk2 were calculated as fraction of the unperturbed scrambled controls. Cells with B-Raf mutation show a linear relation between Erk concentration and phospho-Erk level that is predicted by a mathematical model for a system without feedback (shown as line). Cells with B-Raf wild type show strong robustness in phospho-Erk level corresponding to response coefficients of 0.36 and 0.20 for HCT116 and SW480, respectively. (**D**) Representative western blot images of knockdown experiments in SW480 and HT29. (**E**) Changes in Mek phosphorylation 48 h after treating the cells with scrambled control siRNA or siRNA against Erk1, Erk2 or both isoforms. While B-Raf wild-type cells show a strong increase in phospho-Mek, B-Raf-mutated cells (HT29 and RKO) show no change in phospho-Mek after knockdown. Source data is available for this figure at www.nature.com/msb.

in B-Raf-mutated HT29 cells. However, since in these cells the signal originates from the mutated B-Raf, the feedback has no effect in these cells (see also below).

To exclude that increase of pMek after Erk knockdown is not due to feedback control of Ras in the Ras-mutated cells, we measured Ras activity by an active Ras pulldown assay in cells treated with AZD6244 for 2 h, and compared these with DMSO-treated cells. We find that there is no increase of Ras activation after abrogation of Erk activity in the Ras-mutated cells, excluding negative feedback control upstream or at the level of Ras in these cells harbouring a Ras mutation (see Figure 4B).

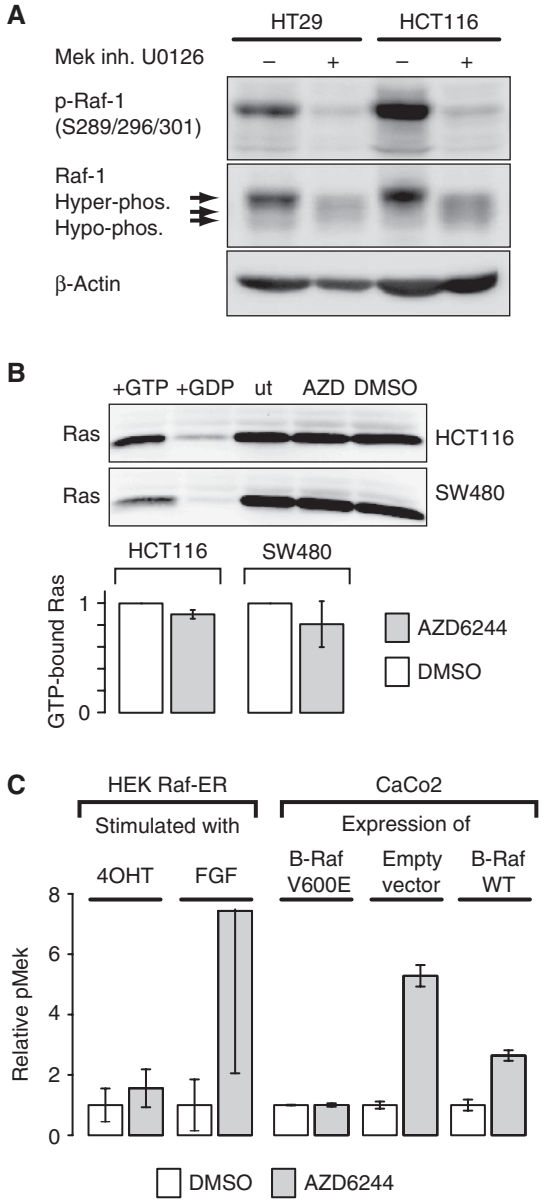

Figure 4 Feedback acts on c-Raf. (**A**) Phosphorylation of Erk-specific phosphorylation sites in Raf-1 changes strongly after Mek inhibition. HT29 and HCT116 cells were treated with MEK inhibitor U0126 or vehicle controls (DMSO) for 24 h, and subsequently analysed using western blotting. Raf-1 displays a prominent phosphorylation at the cluster of ERK phosphorylation sites within the hinge region of Raf-1 in vehicle, but not in U0126-treated cells. Total Raf-1 is accelerated in its electrophoretic mobility after application of U0126. (**B**) Ras activity does not increase in HCT116 and SW480 cells after treating the cells with Mek inhibitor AZD6244, when compared with untreated or vehicle controls. Ras activity was monitored by affinity precipitation using a GST fusion protein containing the Ras-binding domain of Raf-1 and subsequent western blots. GTP and GDP were added to the lysates as positive and negative control for the assay. Representative western blots are shown and quantification relative to DMSO for three assays. (**C**) Analysis of isogenic cells shows that signalling from the catalytic domain of Raf-1 or the V600E mutation in B-Raf diminishes feedback regulation. Left: HEK cells expressing a fusion protein of the catalytic domain of Raf-1 with oestrogen receptor-binding domain show no feedback regulation post-Mek inhibition when stimulated with tamoxifen (4OHT), but show feedback regulation when stimulated with FGF. Right: CaCo2 cells expressing B-Raf V600E show no feedback post-Mek inhibition, while cells with control constructs show strong feedback regulation. Source data is available for this figure at www.nature.com/msb.

To further exclude that the absence of feedback regulation is not due to other differences between the cell lines, we next investigated cell lines with inducible forms of Raf. As a first model system, we experimented with HEK cells that express a fusion protein of the catalytic domain of Raf-1 and the oestrogen receptor-binding domain (Cagnol *et al*, 2006). In these cells, Raf activity can be triggered by adding 4-hydroxytamoxifen (4OHT). The fusion protein lacks the regulatory domain, and is therefore insensitive to feedback regulation. pMek levels in 4OHT-treated cells do not increase strongly after abrogation of Erk activity by the Mek inhibitor (Figure 4C). Thus, MAPK signalling when triggered from Raf that lacks the regulatory domain shows no other prominent feedback. In contrast, if the cells are stimulated by FGF and the Raf–ER fusion protein is not activated by 4OHT, the cells display very strong feedback regulation, as pMek increases strongly once the Mek inhibitor is applied (Figure 4C). As a second model system, we used the colon cancer cell line CaCo2, which we stably transfected with inducible mutated B-Raf V600E, and cells with inducible wild-type B-Raf or an empty expression vector as controls. Again, we measured pMek levels in these cells after applying the Mek inhibitor or vehicle controls. In line with our hypotheses, B-Raf-mutated cells show no feedback regulation, as Mek inhibition does not lead to an increase of pMek, while cells expressing empty vector or overexpressing wild-type B-Raf show strong feedback regulation.

Taken together, several lines of evidence suggest that ppErk levels are controlled by a negative feedback at the level of Raf-1. In order to investigate whether such negative feedback sufficiently explains the observed robustness in ppErk level towards perturbations of the total Erk concentration, we further analysed the data using a mathematical model.

Mathematical analysis shows that feedback is highly amplifying

In order to quantitatively analyse the feedback further, we added feedback inhibition of Mek to the model of Equation (1):

$$\frac{\mathrm{ppErk}}{\mathrm{Erk_T}} = \frac{(k\,\mathrm{pMek}(\mathrm{ppErk}))^2}{1 + (k\,\mathrm{pMek}(\mathrm{ppErk})) + (k\,\mathrm{pMek}(\mathrm{ppErk}))^2}$$
$$\frac{\mathrm{pMek}}{\mathrm{pMek_0}} = 1 - r_1\left(\frac{\mathrm{ppErk}}{\mathrm{ppErk_0}} - 1\right). \qquad (8)$$

The subscript 0 denotes pMek and ppErk levels in unperturbed cells. k denotes the rate constant of phosphorylation by Mek relative to the dephosphorylation rate constant. The parameter r_1 denotes the strength of the feedback, that is, the relative change in phosphorylated Mek upon a relative change in phosphorylated Erk. The parameter r_1 can be interpreted in terms of response analysis, where $|r_1|<1$ denotes weak feedback and $|r_1|>1$ amplifying, strong feedback.

In order to investigate whether the feedback model sufficiently explains the data, we fitted this model to the measured data points for the two cell lines HCT116 and SW480 using a least-squares method. The best model fit can explain the data points well, as shown in Figure 5A. The parameter r_1 was estimated to be 5.4 for HCT116 cells and 2.2 for SW480 cells. Therefore, the model shows that the feedback from Erk to

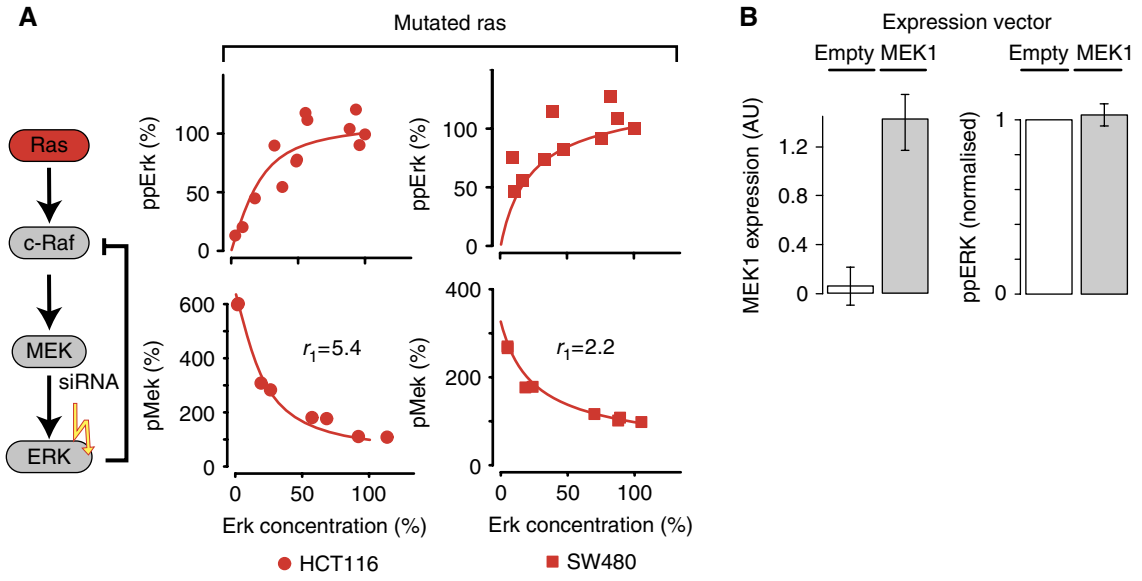

Figure 5 Mathematical analysis shows that the observed phosphorylation of Erk and Mek after knockdown of Erk isoforms can be fully attributed to post-translational feedback to Raf-1. (**A**) Pan-isoform Erk and phospho-Erk levels after knockdown of Erk1 and/or Erk2 were calculated as fraction of the scrambled controls. A mathematical model that includes negative feedback can fit the data. (**B**) Transient expression of Mek1 WT vector in SW480 cells. Even strong overexpression of Mek1 (left panel) does not change the phosphorylation of Erk (normalised to empty expression vector, right panel). Source data is available for this figure at www.nature.com/msb.

Raf is highly amplifying, where a decrease of ppErk by, for example, 10% results in ~20% increase of pMek in SW480 cells and 50% in HCT116 cells.

Taken together, the mathematical analysis shows that negative feedback from Erk to Raf-1 alone is sufficient to explain the observed robustness, and the lack of robustness in B-Raf-mutated cells indicates that other mechanisms are not utilised.

Robustness is not due to saturation of Mek

In the beginning, we hypothesised that competition for the upstream kinase Mek might be a mechanism that causes robustness. Since B-Raf-mutated cells do not show robustness, this mechanism is unlikely. Nevertheless, one could argue that Mek levels in B-Raf-mutated cell lines might be different from Mek levels in B-Raf wild-type cells, and Mek might be only saturated in B-Raf wild-type cells. Therefore, we overexpressed Mek1 in SW480 cells, which have wild-type B-Raf and showed robustness against changes in Erk levels. If robustness was due to limiting amount of Mek, ppErk would necessarily be highly dependent on Mek levels. In contrast, if robustness is solely dependent on negative feedback, Mek overexpression is expected to have only little effect on ppErk.

The results are summarised in Figure 5B. Even strong overexpression of Mek1 does not change the phosphorylation of ppErk; therefore, saturation of pMek can be ruled out as a mechanism that provides robustness in these cells.

Efficiency of small-molecule inhibitors is impaired by strong negative feedback

Several small-molecule compounds have been developed to target the kinase Mek, including the widely used experimental Mek inhibitors U0126 and AZD6244, which is currently tested in phase II clinical trials for different cancers. Both inhibitors bind non-competitively with ATP, thus reaching high specificity for Mek (Yeh *et al*, 2007) with dissociation constants (K_D-values) in the low nanomolar range. Most likely, the inhibitors bind to both active and non-active forms of Mek. Therefore, the action of these pharmacological inhibitors can be included in our model in the following way:

$$\frac{\mathrm{ppErk}}{\mathrm{Erk_T}} = \frac{(k\,\mathrm{pMek}^a(\mathrm{ppErk}))^2}{1+(k\,\mathrm{pMek}^a(\mathrm{ppErk}))+(k\,\mathrm{pMek}^a(\mathrm{ppErk}))^2}$$
$$\frac{\mathrm{pMek}^a}{\mathrm{pMek_0}} = (1-I)\left(1-r_1\left(\frac{\mathrm{ppErk}}{\mathrm{ppErk_0}}-1\right)\right). \quad (9)$$

The superscript a denotes the concentration of active Mek, that is, the non-inhibitor bound, phosphorylated form of Mek. The parameter I denotes the biochemical inhibitor efficiency, which represents the fraction of Mek that is bound by the inhibitor and thereby inactivated. Assuming that the inhibitor binds independent of the phosphorylation state of Mek with a low nanomolar dissociation constant, I is approximated by the inhibitor concentration divided by the Mek concentration.

Using this model, we investigated how the effectiveness of inhibitors is hampered by the presence of the strong negative feedback in B-Raf wild-type cells. To describe the efficiency of Mek inhibitors in feedback-intact cells, we simulate the suppression of Erk phosphorylation at varying inhibitor concentrations using the model above and the kinetic parameters that we have determined for HCT116 and SW480 cells. To compare with B-Raf mutant cells, we repeat the simulation where the feedback strength r_1 is set to zero. The results of this analysis are shown in Figure 6A. The simulations show that the efficiency of Mek inhibitors is strongly reduced through feedback-mediated robustness of the pathway. The model also shows that full inhibition of Mek will result in a

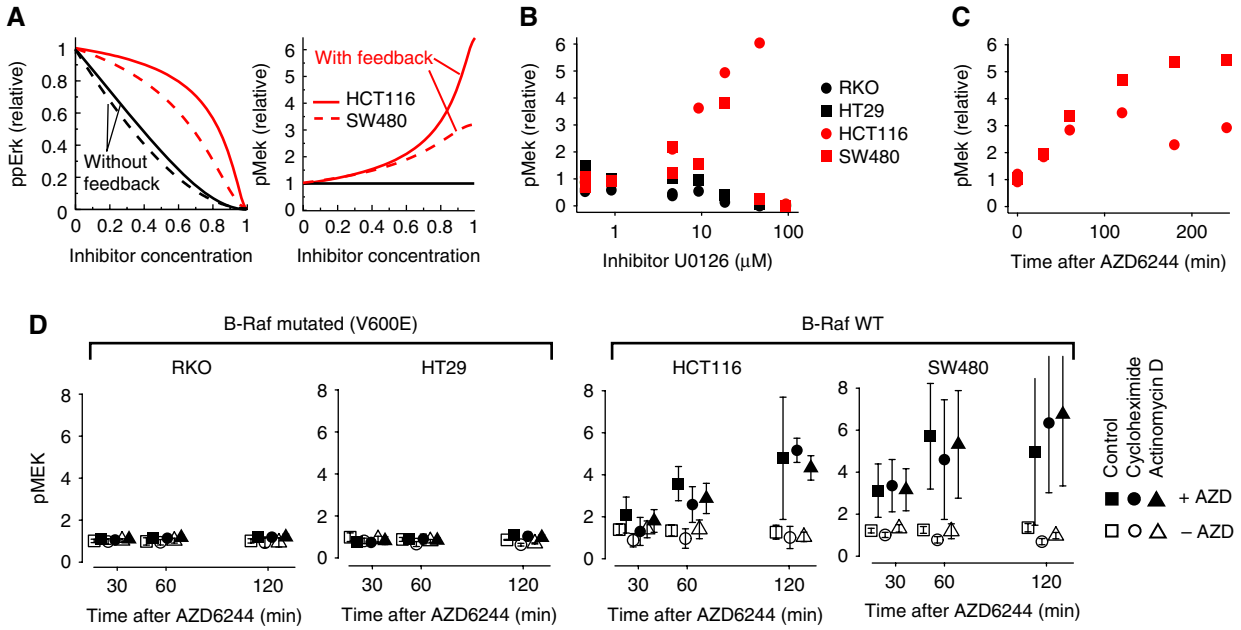

Figure 6 Efficiency of Mek inhibitors is strongly reduced by negative feedback to Raf-1. (**A**) Reduction of phospho-Erk by Mek inhibitors was simulated by a mathematical model for the two cell lines SW480 and HCT116, and compared with the situation in B-Raf-mutated cells, where efficiency of the inhibitors is predicted to be strongly enhanced. In B-Raf wild-type cells, the model predicts a dose-dependent rise of pMek when the inhibitor is applied. (**B**) Experiments show that inhibition of Mek with Mek inhibitor U0126 results in strong increase in Mek phosphorylation in B-Raf wild-type cell lines, while B-Raf V600E cells show no increase. (**C**) A time series experiment after inhibition of Mek with inhibitor AZD6244 shows a rise of pMek within 1 h. At t=0, controls of untreated and DMSO-treated samples are shown. (**D**) Cells are treated with Mek inhibitor AZD6244 (filled symbols) or not (open symbols) for indicated times, and phosphorylation of Mek was measured for four cell lines. In addition, translation inhibitor cycloheximide (circles) or transcription inhibitor actinomycin D was applied. No significant difference between feedback action in cells with non-inhibited gene expression (squares) and cells with inhibited expression can be observed. Shown are mean and s.d. of three replicates relative to untreated samples at 0 time point. Source data is available for this figure at www.nature.com/msb.

strong, three- to six-fold increase of pMek levels in SW480 and HCT116 cells, respectively.

To confirm that the post-translational negative feedback via Raf-1 is active in cells harbouring wild-type B-Raf upon application of the Mek inhibitor, we measured pMek levels 24 h post-inhibition of the pathway. In line with our model, pMek levels rise sharply when Mek is inhibited in B-Raf wild-type cells, contrasted by no increase in B-Raf-mutated cells (Figure 6B). Interestingly, there is a quantitative agreement of the maximal levels of measured phospho-Mek and the predicted maximal levels, with pMek showing a six-fold increase in HCT116 and a three- to four-fold increase in SW480. At inhibitor levels exceeding 20 μM, phospho-Mek levels drop in all cell lines, which cannot be explained by the present model. The mechanism for this drop might be either unspecific effects of the inhibitor, inhibition of Mek phosphorylation at high doses, or indirect effects due to changed phenotype of the cells.

Feedback is fast and does not require translation or transcription

In our analysis, we investigated how the pathway adapts to changes in protein concentrations. The protein concentration of Erk has been shown to change on a timescale larger than cell-cycle time (Cohen-Saidon *et al*, 2009); therefore, we investigated cells 48 h post-perturbation. We found that a single post-translational feedback mediates robustness. As such post-translational feedbacks may act on much faster timescales, we were interested in investigating how quickly the feedback operates. In order to investigate this, we applied the Mek inhibitor AZD6244 to the cells and measured phosphorylated Mek at multiple timepoints after application of the inhibitor. Figure 6C shows that Mek phosphorylation rises within 1 h post-inhibition, suggesting that the feedback acts on the timescale of typical signalling kinetics (<1 h), and may therefore also buffer faster variations within the pathway.

Regulation on such timescale may occur through transcriptional or translational mechanisms. There may, for example, be kinases or phosphatases that are transcriptionally regulated and which regulate Raf or Mek phosphorylation. Other potential regulators may include Sprouty proteins. We therefore investigated whether the action of the feedback requires transcription or translation by investigating the feedback dynamics using generic inhibitors of transcription and translation. We measured phosphorylation of Mek in a time series after treatment with Mek inhibitor AZD6244 either alone or combined with an inhibitor of transcription (actinomycin D) or translation (cycloheximide). Figure 6D shows that, in line with our theory, in B-Raf-mutated cells no change of Mek phosphorylation is observed when the inhibitor is applied. Furthermore, treatment with inhibitors of transcription and translation do not influence pMek levels. In Ras-mutated cells, phosphorylation of Mek increases once the Mek inhibitor is applied, and remains constant under vehicle control. Block of either transcription or translation does not change the increase of phosphorylation of Mek after application of Mek inhibitor in

Ras-mutated cells. Thus, the feedback inhibition does not require synthesis of new proteins. Taken together, these data strongly support that feedback regulation is purely mediated by post-translational mechanisms.

Discussion

Robustness of cellular function in response to physiologically relevant perturbations is a major characteristic of living systems (Stelling *et al*, 2004). In order to analyse how robust specific cellular systems are and which designs have been evolved to provide robustness, it is important to identify the kind of perturbation that a cell witnesses *in vivo* (Kitano, 2004). It becomes clear that noise in the expression of proteins is one of the key perturbations that cellular function has to withstand (Sigal *et al*, 2006). We therefore analysed how the activity of one of the central signal transduction pathways, the MAPK signal transduction pathway, is influenced by changes in protein level. A kinetic model suggested that the active double-phosphorylated form of the terminal kinase in the pathway, Erk, would be linearly dependent on the concentration of the kinase. In stark contrast, we found experimentally that the phosphorylated form is only weakly dependent on the protein concentration.

We identified two mechanisms by mathematical analysis that might account for robustness: saturation of the upstream kinase and negative feedback. The pathway is equipped with a manifold of negative feedbacks at different levels, Erk reduces the activity of various players at different levels upstream in the pathway by phosphorylation, and additionally it induces the expression of deactivating phosphatases (Amit *et al*, 2007; Legewie *et al*, 2008; Kholodenko *et al*, 2010).

We expected that multiple feedbacks would contribute to the observed robustness, and thereby it would be hard to dissect the contribution of each feedback. By analysing cells with different activating mutations in the pathway we found strong robustness to perturbations in Erk levels when Ras is mutated, and no robustness when B-Raf is mutated. To our surprise, this suggested that a single feedback from Erk to Raf is sufficient to explain the observed robustness. While we focused our analysis on feedback to Raf-1, which is the predominantly used isoform in colon cancer cells, additional feedback regulation from Erk to B-Raf has been reported previously (Brummer *et al*, 2003; Rushworth *et al*, 2006; Ritt *et al*, 2010). However, cells with the V600E mutation lack feedback control of B-Raf, as the V600E mutation locks B-Raf into its active conformation (Wan *et al*, 2004). Furthermore, this oncoprotein has bypassed various positive regulatory requirements such as Ras and 14-3-3 binding, which are discussed to be modulated by ERK-mediated feedback (Brummer *et al*, 2006; Rushworth *et al*, 2006; Ritt *et al*, 2010). Furthermore, the active conformation renders B-Raf V600E also resistant against negative feedback regulators such as Sprouty proteins (Tsavachidou *et al*, 2004; Brady *et al*, 2009).

Interestingly, the post-translational feedback from Erk to Raf-1 is relatively fast and acts on a timescale of 1 h; thus, it may also confer robustness to short-term signalling. It has recently been shown that this feedback has profound effects on the robustness of this signalling pathway 20 min post-stimulation, suggesting that the pathway acts as a negative feedback amplifier (Sturm *et al*, 2010).

We found that transcriptional feedback, for example via phosphatases, is not involved in mediating robustness. This is surprising, as intuitively one might think that variability within the pathway that changes within days may be buffered by slow feedback, which seems however not to be the case. Therefore, the role of these feedbacks in the pathway remains puzzling. One reason might be that strong transcriptional feedback introduces further stochasticity.

Our analysis shows that feedback to Raf-1 is sufficient for robustness. However, we cannot exclude that other post-translational feedbacks do also contribute to robustness. One could speculate that the other post-translational feedbacks are fail-save mechanisms, that is, that they could compensate for the loss of the feedback domain in Raf-1. Another possibility is that they actually mediate cross talk as the other feedback targets, for example, SOS, Src and EGFR, also activate parallel pathways. In addition, the transcriptional negative feedbacks, which are a wide-spread phenomenon (Legewie *et al*, 2008), may be more important in fine-tuning the length of response in transient signalling to reduce noise in target gene expression (Bluthgen, 2010). Also, they may have an important role in buffering strong overactivation (Haigis *et al*, 2008).

In our study, we concentrated on steady-state signal transduction. Such long-term signalling via Erk is important for example in the context of G1-S transition (Yamamoto *et al*, 2006; Meloche and Pouyssegur, 2007). It is also interesting to study the consequences of heterogeneity of Erk levels on short-term signalling, that is, after stimulation with a growth hormone. A recent analysis has shown that the feedback to Raf-1 provides also robustness against inhibition of Mek for short-term signalling (Sturm *et al*, 2010). However, since phosphorylation levels are generally much higher after stimulation, it may be that the feedback is not sufficient and may be to slow to compensate decreased Erk protein levels. Thus, it may be that short-term signalling is very heterogeneous, but long-term signalling is not. In line with this theory, an imaging study has shown that nuclear accumulation in the first phase after stimulation is proportional to basal expression of Erk (Cohen-Saidon *et al*, 2009). These authors suggest that the readout for transient, short-term signalling is fold-change rather than absolute values, and that the downstream early transcriptional network interprets fold-change rather than absolute values through feed-forward loops.

As a consequence of the negative feedback, we quantitatively predicted that targeted Mek inhibitors would be more potent in cells with B-Raf mutation than in cells without B-Raf mutation. There are several studies that investigate an association between successful inhibition of cell growth by non-ATP competitively binding Mek inhibitors with B-Raf mutation status. A recent study showed that Mek inhibitors in B-Raf (V600E)-mutated cells show similar success as in K-Ras-mutated colon cancer cells (Yeh *et al*, 2009). In contrast, other studies show that in cancers such as non-small cell lung cancer and thyroid cancer a B-Raf V600E is required for success of Mek inhibitors (Solit *et al*, 2006; Friday *et al*, 2008; Leboeuf *et al*, 2008). Our results suggest that the differences between cells with mutated and wild-type B-Raf will not be qualitative, but more a quantitative difference. Our model predicts that in

all cell lines that depend on MAPK signalling, Mek inhibition at higher doses will abrogate Erk signalling. However, due to loss of negative feedback, B-Raf-mutated cells will respond at lower doses.

Our study highlights the importance of understanding the mechanisms by which signal transduction pathways buffer gene expression noise. These evolved robustness mechanisms will largely effect how the signal transduction network reacts upon targeted inhibition. In the case of the MAPK signalling pathway, robustness seems to be mediated by one feedback alone. Therefore, once the feedback is broken, the system loses robustness and can be readily modulated by low concentrations of targeted inhibitors. In contrast, if the feedback is intact, inhibition of the pathway is inefficient. This finding explains why Mek inhibition has shown little success in the past in cancer treatment. However, it also shows that a subgroup of patients with B-Raf mutation will likely benefit, and that due to the robustness of the healthy cells that have no B-Raf mutation side effects might be minimal. We believe that analysing robustness of other signalling pathways in a similar way will be the key to devise efficient targeted interventions for these, and will unveil which mutations in the pathway will break robustness and thereby open the door for efficient intervention.

Materials and methods

Cells and cell culture

The cell lines SW480, HCT116, HT29 and RKO were obtained from the ATCC (American Type Culture Collection, UK). LIM1215 was kindly provided by Professor John Mariadason (Ludwig Institute for Cancer Research, Austin Hospital, Melbourne, Australia). Cell lines RKO and LIM1215 were maintained in DEMEM (Dulbecco's Modified Eagle's Medium, Lonza) supplemented with 10% fetal calf serum, 1% ultraglutamine and 1% penicillin/streptomycin. SW480, HCT116 and HT29 were cultured in L15 medium (Leibovitz's Medium, Lonza) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. All cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. In order to establish an inducible expression system for B-Raf, CaCo2 cells, a kind gift of Professor Thomas Brabletz (Freiburg), were transfected with *Ahd*I-linearised pWHE 644, which was kindly provided by Dr Christian Berens (Erlangen). This vector encodes the doxycycline-inducible system components rtTA and rtTS (Danke *et al*, 2010). Following selection with puromycine (8 μg/ml), a stable pool of Caco2-tet cells expressing the rtTA and rtTS was subsequently transfected with *Ahd*I-linearised pTET/HAhBRAF-IRES-GFP-bsr vectors and selected with blasticidine S (5 μg/ml) and puromycine (5 μg/ml). The latter vector will be described in detail elsewhere (Herr *et al*, under revision) and contains the bi-cistronic HA-BRAF-IRES-GFP expression cassette from pMIG/HAhBRAF (Eisenhardt *et al*, 2010) that encodes for hemaglutinine (HA)-tagged human B-Raf and green fluorescent protein (GFP). Cells transfected with pTET/-IRES-GFP-bsr respond to doxycycline treatment with expression of GFP and serve as negative control. HA-B-Raf expression was induced by addition of doxycycline hyclate (Sigma; 2 μg/ml final concentration). HEK Raf-ER cells were used for stimulation experiments. These cells express the oestrogen receptor–Raf-1 fusion molecule, and were described previously (Cagnol *et al*, 2006).

Transient transfection assays

For siRNA transfection, cell lines were plated 24 h before siRNA knockdown. Cells were either mock-transfected, scrambled-transfected (ON-TARGETplus Non-targeting Pool negative control siRNA with at least four mismatches to any human, mouse or rat gene; Thermo Scientific Dharmacon) or transfected with 50 nM Erk1 (siRNA pool using the following sequences from Invitrogen: MAPK3HSS108538 GGAAGCCAUGAGAGAUGUCUACAUU, AAUGUAGACAUCUCUCAUGGCUUCC; MAPK3HSS108539 GCAUUCUGGCUGAGAUGCUCUCUAA, UUAGAGAGCAUCUCAGCCAGAAUGC; MAPK3HSS108540 CCUGCUGGACCGGAUGUUAACCUUU, AAAGGUUAACAUCCGGUCCAGCAGG), Erk2 (MAPK1HSS1085535 GCCGAAGCACCAUUCAAGUUCGACA, UGUCGAACUUGAAUGGUGCUUCGGC; MAPK1HSS1085536 UCACACAGGGUUCCUGACAGAAUAU, AUAUUCUGUCAGGAACCCUGUGUGA; MAPK1HSS1085537 GGGCUACACCAAGUCCAUUGAUAUU, AAUAUCAAUGGACUUGGUGUAGCCC) siRNA or both for Erk1/2 knockdown, further using lipofectamine 2000 or RNAi-Max (both from Invitrogen) according to the manufacturer's instructions. Briefly, 7.5 μl (15 μl for scramble) siRNA and 7.5 μl transfection reagent were combined in a total of 500 μl Opti-MemI + Glutamax-I (GIBCO). The mix was incubated for 20 min at room temperature before adding the complexes dropwise to the cells. After 24 h, cells were transfected for a second time, followed by a medium change after 3–4 h. RNA and protein extracts were prepared 48 h after second transfection. siRNA experiments were repeated in minimum three times. For overexpression of Mek1, SW480 cells were transiently transfected with pMEV2HAMek1-WT vector or empty pcDNA3 as control (Biomyx Technology) using lipofectamine 2000 (Invitrogen). Cells were harvested and lysed 24 h post-transfection.

RNA isolation and quantitative RT–PCR analysis

RNA was isolated from cells which were transiently siRNA transfected (48 h post-transfection) or treated 24 h with 20 μM U0126 using the RNeasy-mini-kit (Qiagen) according to the supplier's protocol. Quantitative real-time PCR analysis was performed using a StepOnePlus 96-well format Light-Cycler apparatus (Applied Biosystems). Experiments were run and analysed with the StepOne 2.0 software according to the manufacturer's recommendations. Synthesis of double-stranded DNA during the PCR cycles was visualised with SYBR Green using QuantiTect SYBR Green RT–PCR kit, (Qiagen). The following QuantiTect primer assays were used: Hs_DUSP1_1_SG, Hs_DUSP2_1_SG, Hs_DUSP4-va.1_SG, Hs_DUSP5_2_SG, Hs_DUSP6_1_SG, Hs_DUSP7_1_SG, Hs_DUSP8_1_SG, Hs_DUSP9_1_SG, Hs_DUSP10_va.1_SG and Hs_DUSP16_1_SG. All samples were run in triplicates and two biological replicates. Target expression was quantified relatively to actin (Hs_ACTA1_1_SG) expression. We measured standard calibration curves to confirm that the PCRs had full efficiency.

Immunoblotting

Protein extracts of cells were prepared by incubation with sodium dodecyl sulphate (SDS)-cell lysis buffer (10% SDS, 1 M Tris–HCl pH 7.5, EDTA 0.5 M pH 8) or cell lysis buffer (Bio-Rad). Reagents for SDS–polyacrylamide gel electrophoresis (PAGE) and western blotting were obtained from Bio-Rad Laboratories (Richmond, CA, USA) and Carl Roth (Karlsruhe, Germany). Electrophoresis was performed and lysates were transferred onto nitrocellulose membranes (Schleicher & Schüll). Unbound protein sites were blocked with 1:1 Li-COR buffer in phosphate-buffered saline. Thereafter, specific proteins were detected by incubation with primary antibodies diluted in 1:1 Li-COR buffer/phosphate-buffered saline containing 0.1% Tween-20 (PBST) overnight at 4°C followed by near-infrared dye labelled secondary antibodies. The following antibodies were used: rabbit anti-human P-Erk1/2 (phospho-p44/42 MAPK Thr202/Tyr204, Cell Signaling Technology, 1:500), rabbit anti-human Erk1/2 (p44/42-MAPK, Cell Signaling Technology, 1:500), mouse anti-human Actin (Millipore, 1:5000 or Santa Cruz Biotechnology, 1:2000), rabbit anti-phospho-Raf-1 (S289/S296/S301, Cell Signaling Technology, 1:1000), anti-pan-Ras (Millipore, 1:200), rabbit anti-human Raf-1 C-12 (Santa Cruz Biotechnology, 1:750), rabbit anti-human Tubulin (Cell Signaling Technology, 1:2000) or mouse anti-human GAPDH (Ambion, 1:10 000). Membranes were scanned using Li-COR Odyssey. The bands were quantified by determining the integrated density using ImageJ software (http://rsbweb.nih.gov/ij/) and subtracting the mean background intensity multiplied by the area of the band. All intensities were

normalised to a control (Tubulin, Actin or GAPDH) and expressed relative to scramble controls. Since the pErk antibody detects the highly conserved phosphorylation domain it displays equal affinity to ppErk1 and ppErk2 (Lefloch *et al*, 2008). Therefore, the sum of ppErk1 and ppErk2 bands is taken as total phosphorylation of Erk. Since the Erk1/2 antibody shows different affinities to the two isoforms, this affinity needs to be calibrated (Lefloch *et al*, 2008). We determined the ratio of affinities to Erk1 and Erk2, respectively, by comparing the ratio of ppErk1/ppErk2 bands and Erk1/Erk2 bands from unperturbed cells (embracing all conducted experiments). We found that the antibody displayed a lower affinity to Erk2 than to Erk1 (0.29 ± 0.09). Therefore, the amount of total Erk was calculated by scaling the intensity of the Erk1 band with the factor 0.29, and adding it to the unscaled intensity of the respective Erk2 bands. The data confirming the effect of the MEK inhibitor on the feedback phosphorylation of Raf-1 were generated by western blotting using a Fuji-LAS 4000 device as described previously (Eisenhardt *et al*, 2010).

Bio-Plex assay

Cells were transfected with siRNA or treated with different concentrations of U0126 (Promega), 1 μM AZD6244 (Selleck Chemicals LLC), 10 μg/ml Cycloheximide (Calbiochem), 5 μg/ml Actinomycin D (Sigma), 50 nM 4OHT or 0.005 μg/ml FGF2 (Preprotech). After incubation, lysates were collected and the level of phospho-protein expression was analysed with the Bio-Plex Protein Array system (Bio-Rad, Hercules, CA) using beads specific for P-Mek1 (S217/S221) according to the manufacturer's instructions. Briefly, samples were washed with PBS and lysed with cell lysis buffer (Bio-Rad). Lysate protein concentration was determined with BCA (bicinchoninic acid) method. The beads and detection antibodies were diluted 1:5. For acquiring data, the Bio-Plex Manager software was used according to the manufacturer's instructions.

Ras activity assay

For the Ras activity assay, the active Ras pulldown and detection kit from Thermo Scientific was used according to the manufacturer's instruction. Briefly, cells were plated 24 h prior treatment with AZD6244 for 2 h. Cells were collected and lysed, and the lysate incubated with GST-Raf-1-RBD, a GST fusion protein that contains the Ras-binding domain of Raf-1. Using an immobilised gluthatione disc, the active Ras was precipitated and detected by western blot using a Ras-specific antibody.

Mathematical modelling

The numerical simulations of models in Equations (8) and (9) were done in Matlab, Version R2007b. Model equations for ppErk were solved numerically using matlab function fzero. Parameter estimation was performed by minimising the squared residuals using lsqnonlin. Analytical calculations in this study were partially done using Mathematica algebraic software.

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (www.nature.com/msb).

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Author contributions: RFG designed and performed the experiments with Erk knockdowns, Mek overexpression, Ras activity, RT–PCR and contributed to paper writing; FW designed and performed the modelling, data analysis and wrote paper; AS designed and performed the Luminex experiments; NS performed experiments; RH cloned constructs, established and characterised CaCo2 cell lines, MEK inhibitor and knockdown experiments; SB performed all western blotting associated with Raf-1 shift; TB contributed to study design, data analysis and paper writing; CS contributed to analytical discussions, experimental design and paper writing; NB directed and designed the study, led the data analysis, wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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