MAPK kinase signalling dynamics regulate cell fate decisions and drug resistance
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The RAS/RAF/MEK/MAPK kinase pathway has been extensively studied for more than 25 years, yet we continue to be puzzled by its intricate dynamic control and plasticity. Different spatiotemporal MAPK dynamics bring about distinct cell fate decisions in normal vs cancer cells and developing organisms. Recent modelling and experimental studies provided novel insights in the versatile MAPK dynamics concerted by a plethora of feedforward/feedback regulations and crosstalk on multiple timescales. Multiple cancer types and various developmental disorders arise from persistent alterations of the MAPK dynamics caused by RAS/RAF/MEK mutations. While a key role of the MAPK pathway in multiple diseases made the development of novel RAF/MEK inhibitors a hot topic of drug development, these drugs have unexpected side-effects and resistance inevitably occurs. We review how RAF dimerization conveys drug resistance and recent breakthroughs to overcome this resistance.

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This review comes from a themed issue on Multi-protein assemblies in signaling
Edited by Arun K Shukla and Shoshana J Wodak

Introduction
The mitogen-activated protein kinase (MAPK) cascades have been in the limelight of research for over 25 years due to their involvement in cell proliferation, differentiation, survival/apoptosis, and motility. This scientific interest is also practical, because deregulation of MAPK signalling is a feature of major human diseases and developmental disorders [1,2]. The MAPK signalling cascades are activated by a plethora of external cues through a multitude of membrane receptors, including receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs). All MAPK cascades have the evolutionary conserved three-tier architecture where kinases sequentially phosphorylate and activate each other, whereas phosphatases dephosphorylate these kinases [3*]. The initiating kinases (such as RAF1/CRAF and BRAF) in the extracellular regulated kinase 1/2 (ERK1/2) cascade are commonly activated by RAS small G-proteins. The components of the RAS/RAF/MEK/ERK cascade are frequently mutated in cancer [4**] and are hot targets for an ever increasing number of anti-cancer drugs [5,6*].

Although the RAS/RAF/MEK/ERK pathway has a predominantly linear architecture, RAS and ERK are signalling hubs that have tens and hundreds of effectors and substrates, respectively. Changes in these interactions can dramatically change the ERK cascade temporal dynamics [7]. Different ERK dynamics were shown to trigger distinct cell decisions. For instance, in rat adrenal pheochromocytoma PC12 cells a transient activation of ERK induced by epidermal growth factor (EGF) results in cell proliferation, whereas sustained ERK activation by nerve growth factor (NGF) induces differentiation [8]. Subsequently, Bastiaens and colleagues showed that distinct cell decisions are explained by dynamic changes in the ERK cascade topology where EGF stimulation elicited negative feedback, whereas NGF-induced positive feedback, imposed on the backbone of the same three-tier cascade structure [9]. Yet, recently Pertz and colleagues demonstrated that repeated 3 min pulses of low EGF concentration resulted in prolonged ERK activation and PC12 cell differentiation, whereas pulses of high EGF induced a more transient ERK response and PC12 cell proliferation similar to sustained EGF stimulation [10*]. The fact that the ERK cascade inputs of different frequency and amplitude rewire cell fate raised intriguing questions about the design and timescales of multiple feedforward and feedback regulations in the ERK network. Answering these questions requires a careful probing of the RTK/RAS/ERK network circuitry, uncovering timescales of major regulations, and the use of computational models. Here we present a brief overview of the current research efforts in the field, emphasizing a combined use of modelling and experiments as a tool to advance both the fundamental understanding of the control of ERK signalling and therapeutic applications.
**Versatile ERK dynamics: adaptive and sustained signalling, switches and oscillations on different timescales.**

In many cells, the RAS/ERK cascade exhibits a transient, adaptive response to growth factor (GF) stimulation due to negative feedback loops from ERK to SOS and BRAF/CRAF, and the negative transcriptional feedback via ERK-induced expression of the DUSP family phosphatases on a longer timescale. However, in other cells, the ERK response is sustained indicating that positive feedback and feedforward regulations reverse the effect of multiple negative feedbacks [11]. Positive feedback loops can also induce switch-like, bistable responses, where there is a threshold stimulus magnitude, and ERK is active until the stimulus decreases to much lower than threshold nearly basal values [9,12].

A main role of negative feedback is to confer resistance to perturbations of cascade components within the feedback loop. Due to several negative feedbacks, the ERK cascade can operate as a negative feedback amplifier, and this circuitry resists MEK inhibition by anti-cancer drugs [13]. When a negative feedback becomes too strong, it induces damped or sustained oscillations [14]. A combination of positive feedforward regulations by RTKs and positive and negative feedbacks can explain the ERK oscillations observed on remarkably different timescales from minutes to several hours [10*,15,16**].

Mathematical modelling helps to elucidate the intricate network dynamics [17]. Recently, the feedforward and feedback regulation of the RTK/RAS/ERK network and the dynamics of ERK responses were probed in live single PC12 cells using microfluidic devices to deliver GF input stimulation with precisely defined kinetics [10*]. Whereas sustained EGF/NGF stimulation largely confirmed previous observations (while uncovering a remarkable heterogeneity of signalling in individual cells), ERK responses to GF pulses of different frequency and amplitude showed unexpected signalling and biological outcomes. Mathematical modelling proved that known negative and positive feedback regulations could not explain the observed ERK dynamics. For instance, an EGF stimulatory regime of 3 min pulses/10 min intervals between pulses led to successive ERK pulses where the amplitude decayed much less for low than for high EGF concentrations. Since the ERK activity peaks in response to the initial EGF pulse were identical for both low and high EGF doses, ERK-induced negative feedbacks alone could not account for these observations. A dynamic model recapitulated these observation assuming that the negative ERK influence must be modulated by a feedforward signal from the EGF receptor, which was differentially activated by low and high EGF doses [10*].

A high-dosage, single NGF pulse of 10 min induced sustained ERK activity in a cell subpopulation, suggesting the presence of bistability brought about by a positive feedback from ERK, as previously reported [9]. In contrast, short 3 min NGF pulses of both high and low doses, and low dose 10 min NGF pulse elicited transient, adaptive ERK activity profiles throughout the cell population. This implies a slightly delayed and threshold activation of NGF-evoked positive feedback that occurred only if ERK activity exceeds certain levels of amplitude and duration. Since the first peak-response of NGF was similar for all pulsed NGF stimulation conditions, the data also suggested the existence of additional feedforward regulation of the ERK positive feedback strength [10*]. Figure 1 illustrates a kinetic scheme of a minimal mathematical model that was able to reproduce all these experimental observations, including heterogeneity of responses, which was more pronounced for NGF due to positive feedback from ERK.

**Figure 1**

A simplified diagram of feedforward and feedback regulation of the RTK/RAS/ERK network activated by EGF and NGF in PC12 cells. Modified from Ref. [10*].

**Oncogenic alterations in RAS/RAF/MEK/ERK signalling**

Many human cancers present hyperactivation of the MAPK pathway, most commonly through mutations in RAS, BRAF, CRAF, or MEK1/2 [4**]. Components of this pathway are therefore attractive targets for drug development [5,6*,18].

Germline mutations in genes encoding MAPK pathway components are associated with a group of developmental disorders known as RASopathies or RAS/MAPK syndromes [19,20]. Biochemical studies of these mutants as well as structural analysis and network-level data...
suggestion that MAPK pathway activation in RASopathies is quantitatively, rather than qualitatively, different compared to cancer-related mutations [1].

About 30% of all human cancers, including about 90% of pancreatic cancer and 45% of colorectal cancer cases, present activating mutations in RAS family genes (HRAS, KRAS, and NRAS) [21], with KRAS being the most frequently mutated isofrom [22]. Since the discovery of RAS as an oncogene over 30 years ago [23,24,25,26], no clinically effective inhibitor has yet been developed, giving RAS a reputation as ‘undruggable’. Recently, however, a renewed interest in RAS as drug target has arisen [27,28,29]. Promising novel approaches include targeting the RAS-SOS complex [30,31,32], inhibiting the interaction with downstream effectors [33], interfering with membrane localization [34], and the development of mutation-specific small molecule inhibitors [35,36]. A novel small molecule, Rigosertib, was able to inhibit MAPK pathway activation through interaction with the RAS-binding domains (RBDs) of RAS-effector proteins, including RAF [37]. Rigosertib has entered clinical phase 3 studies validating the strategy of targeting protein-protein interactions as a viable approach to target proteins that previously were considered ‘undruggable’.

Among the three RAF kinase family members, BRAF is the most frequently mutated isofrom in human cancers [4**,38], with particular high prevalence of mutations (>50%) in melanoma [39**] and papillary thyroid carcinoma [40]. Over 50 mutations have been identified in BRAF — related to cancer and RASopathies — most of which are located in the kinase domain (Figure 2) [4**,41**]. Characterization of 22 oncogenic BRAF mutants revealed that the majority of these directly increase BRAF kinase activity, while others activate ERK signalling through enhanced dimerization with and transactivation of wildtype CRAF [42].

BRAF has become an important therapeutic target in cancer therapy [18,43]. While small molecules targeting BRAF are ATP-competitive inhibitors, they can employ different mechanisms: Class I inhibitors (e.g. Vemurafenib/PLX4032) recognize the active (‘DFG-in’) conformation [44], whereas class II inhibitors (e.g. Sorafenib/BAY-439006) bind to and stabilize the inactive (‘DFG-out’) conformation [44]. As 98% of BRAF mutations are found at position V600, with V600E being the most common one [41**], mutation-selective BRAF inhibitors such as Vemurafenib [44,45] and Dabrafenib/GSK2118436 [46] were developed. RAF inhibitor treatment initially leads to high response rates in patients with BRAF mutant melanoma, but drug resistance develops within 6–9 months in the majority of patients [47]. Different mechanisms have been described (reviewed in [48]), most of which involve reactivation of the MAPK pathway. These include secondary mutations in NRAS [49] or MEK1 [50], a BRAF(V600E) splice variant with enhanced dimerization [51], the expression of an alternative MEK kinase [52], or overexpression of RAS, BRAF, CRAF or RTKs [47]. In addition, other cellular processes, including TGFβ signalling, chromatin modifying enzymes, and the Mediator complex, have recently been linked to drug resistance in high-throughput screens and could possibly lead to novel therapeutic strategies [53,54,55*,56].

While less common, activating mutations in MEK1 and MEK2 occur in several types of cancer, including melanoma [57] and lung adenocarcinoma [58]. MEK1 and MEK2 mutations also contribute to drug resistance to RAF and MEK inhibition in BRAF mutant melanoma [50,59,60,61,62]. While allosteric MEK inhibitors have suffered from limited clinical success, their combination with RAF inhibitors (Trametinib/GSK1120212 or Cobimetinib/GDC-0973 with BRAF inhibitors Dabrafenib or Vemurafenib) has abolished the side effects of therapy induced skin tumours, delayed drug resistance, and improved survival in patients with BRAF mutant melanoma [63,64]. Further inhibitors are currently in development and clinical trials [65].

Why kinase dimerization conveys drug resistance: thermodynamics has the answer

Targeted cancer therapies are hampered by intrinsic and acquired drug resistance. As mentioned, although ATP-competitive RAF inhibitors are used in the clinic, these drugs ‘paradoxically’ activate the ERK pathway, especially in BRAF wild-type cells [41**,66]. A culprit of this unexpected activation is heterodimerization of RAF kinases. In RAF dimers, a kinase-dead or inhibited RAF protomer allosterically activates the other inhibitor-free protomer, driving ERK signalling. Paradoxical ERK activation by RAF inhibitors in melanoma patients causes both drug resistance and clinical side-effects, including the frequent incidence of keratoacanthomas and squamous-cell carcinomas [5]. Recent efforts have produced RAF inhibitors that evade this paradoxical ERK activation [67,68], although the reason remained obscure why within RAF dimers only one protomer binds drug.

Kholodenko showed that drug resistance caused by kinase dimerization results from fundamental thermodynamic principles [69]. The inhibitor binding to one kinase monomer allosterically facilitates its dimerization with a free monomer. In this constellation, one protomer that is drug-bound allosterically activates the other, drug-free protomer. Mathematical models show the significant accumulation of such dimers that harbour drug-bound and free protomers are active and convey resistance [69]. Thermodynamic laws further imply that two protomers in a dimer may acquire different drug affinities. This emergence of different drug affinities between monomers and protomers in a kinase dimer has long been enigmatic. Using different RAF mutants it was shown that binding of
RAF inhibitor to one protomer in a dimer commonly reduces the affinity for binding the drug to the second protomer in the dimer [70]. Instructively, mathematical models show that while for any inhibitor that facilitates kinase dimerization, the first inhibitor molecule always binds to a dimer with the lower affinity than to a monomer conferring resistance, it is still thermodynamically feasible that the affinities of the first and the second drug molecule binding to protomers in a dimer will be equal [69]. Confirming these model-based predictions, only one inhibitor, BGB659, from a large panel of currently used RAF inhibitors showed comparable binding affinities for both protomers in a RAF dimer [70]. However, as predicted by the model [69], BGB659 also showed paradoxical activation of the ERK cascade, most pronouncedly in RAS-mutant melanoma cell lines [70]. Likewise, this model predicted that pan-RAF inhibitors that bind equally well to CRAF and BRAF are much more effective than specific BRAF inhibitors [69], which is in line with recent experimental findings [67, 71]. Peng et al. described a new pan-RAF inhibitor, LY3009120, that was effective against RAS or BRAF mutant tumour cells in vitro and in vivo while showing a minimal paradoxical ERK pathway activation, despite increasing RAF dimerization [71]. Given the low half-maximal inhibitory dose, IC50 of 5–10 nM, computer simulations [69] show that following paradoxical pathway activation at very low doses, a further increase in LY3009120 effectively inhibits the total RAF and ERK
activities, which explains the data reported by Peng et al. [71*].

Computational models of protein and inhibitor interactions not only predict drug dose-response dependencies but also suggest new ways to overcome drug resistance [69]. These models demonstrate that two inhibitors, aimed at the same kinase target and ineffective on their own, can abolish drug resistance when combined (Figure 3). This unexpected finding is an elegant demonstration how dynamic systems biology models can streamline drug development [69].

Another important insight emerging from studying signalling dynamics was the explanation for the conundrum that dimerization can confer drug resistance to kinase inhibitors. Borne out with RAF kinase as paradigm, this mechanism likely is widespread. Numerous kinases are allosterically regulated by dimerization. For instance, in the case of Janus kinases (JAKs) dimerization induced resistance to JAK inhibitors in patients with myeloproliferative tumours [72]. Having recognized the mechanism enables the design of new inhibitors or inhibitor combinations that overcome this type of resistance.

Conflict of interest
The authors declare that they have no conflicts of interest.

Acknowledgements
This work was supported by the European Union FP7 grants “PRIMES” (No. 278568) and “SynSignal” (No. 613879) and H2020 SmartNanoTox (No. 686098). We would like to thank Drs. Fey and Nguyen for their help with the preparation of graphical material.

References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- - of outstanding interest


Novel cellular pathways potentially involved in drug resistance were identified in a genome-scale knockout screening using CRISPR/Cas9 technology.


Developed a pan-RAF inhibitor that binds all three RAF family members (A-, C- and BRAF) with very low IC50 and effectively inhibited ERK activation in RAS or BRAF mutant tumor cells in vitro and in vivo.
