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REVIEW

It takes two to tango – signalling by dimeric Raf kinases

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Raf kinases function downstream of Ras proteins to activate the MEK-ERK pathway which is deregulated in a large number of human cancers. Raf inhibitors are clinically highly effective for the treatment of cancer and melanoma in particular, but have unexpected side effects that include a paradoxical activation of the ERK pathway. These effects seem to be related to the heterodimerization of Raf-1 and B-Raf kinases. Here, we discuss the role of Raf dimerization as part of the physiological activation mechanism of Raf kinases, the mechanism of Raf dimerization induced by drugs, and the implications of dimerization for drug therapies targeting Raf kinases.

Introduction

Raf is a central component of the classic MAPK/ERK (mitogen-activated protein kinase/extracellular signal regulated kinase) pathway, which uses a three-tiered kinase cascade, Raf-MEK-ERK, to mediate the effects of different extracellular stimuli on various cellular processes including proliferation, differentiation, transformation, motility and apoptosis.^{1, 2} While vertebrates express three Raf family members, Raf-1, A-Raf and B-Raf, lower eukaryotes have only one Raf gene, most closely related to B-Raf. Interestingly, B-Raf also has the highest MEK kinase activity suggesting that during evolution kinase-independent functions may have become prevalent. The discovery of B-Raf mutations in human cancer³ and the frequent hyperactivation of the Raf pathway due to Ras mutations or aberrations of growth factor receptors have made this pathway a prime target for new cancer drugs.^{1, 2, 4}

The Raf activation/deactivation cycle is highly complex, but despite 30 years of research still incompletely understood. As the current state of knowledge is well described in recent reviews^{1, 5, 6}, we only present aspects that are pertinent to the topic of this review. In quiescent cells Raf proteins exist in an inactive conformation maintained by autoinhibitory interactions between the N-terminal regulatory and the C-terminal catalytic domains. This inactive conformation is stabilized by binding of 14-3-3 dimers to conserved phosphoserine residues within the N- and C-terminus. Main Raf activators are Ras GTPases, which are activated by many growth factor receptors. Their active Ras-GTP forms bind to the RBD (Ras binding domain) of Raf with high affinity, displacing 14-3-3 from the N-terminal binding site and presumably inducing conformational changes that initiate activating modifications such as the phosphorylation of activating sites in the N-region and activation loop (Fig. 1A). A host of recent papers has added dimerization as key mechanism of Raf activation in response to physiological, pathological and pharmacological signals. The first suggestion that Raf

dimerization is involved in Raf activation came from experiments where Raf-1 proteins were artificially dimerized^{7, 8}, but it took another 5 to 10 years⁹⁻¹¹ before dimerization was recognized as physiological part of the Raf activation cycle. In this review we will discuss the role of dimerization in Raf activation and oncogenic signalling with a focus on the implications for drug treatment of cancer and the design of combination therapies.

Regulation of Raf dimerization by Ras family proteins

Growth factor stimulation¹¹ and the expression of activated, mutant Ras induce the dimerization of Raf proteins.⁸⁻¹⁰ The mechanism is unclear, but it was suggested that binding of Ras-GTP induces an open conformation in Raf that makes the dimerization surface available for interaction. The finding that Ras also forms dimers¹² raises the intriguing possibility that Ras binding may directly promote Raf dimerization. Interestingly, this relationship is mutual, as artificially induced Raf dimerization also increases the nanoclustering of Ras at the plasma membrane, suggesting that the RBD domains of two Raf protomers crosslink Ras proteins during dimerization.¹³ The dimerization triggers phosphorylation of Raf-1 on S338, a site in the N-region essential for activation¹³⁻¹⁵, and it has been suggested that S338 is trans-phosphorylated by dimerized Raf itself. These data indicate a two-step model of Raf dimerization with a positive feedback. The initial binding of Ras dimers to Raf recruits Raf to the membrane and relieves the close conformation of Raf thereby priming Raf for dimerization. The subsequent phosphorylation of the N-region, either by Raf itself or other kinases, strengthens the Ras/Raf binding¹⁶ and stabilizes Raf dimerization, which in turn stabilizes Ras nanoclusters and Ras mediated Raf activation (Fig. 1A).¹³

On the other hand, the Ras family GTPases Rheb and DiRas3 inhibit Raf dimer formation and antagonize Ras-GTP induced Raf activation. Although the outcome of Rheb- and DiRas3-mediated

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Fig. 1 Raf dimerization. (A) Dimerization as part of the physiological Raf activation mechanism. In quiescent cells, Raf is phosphorylated on the N-terminal and C-terminal 14-3-3 binding sites (pS259/pS621 in Raf-1; pS365/pS729 in B-Raf), and sequestered in the cytosol by binding to 14-3-3 dimer that maintains the closed inactive conformation. Activated receptor tyrosine kinases (RTKs) recruit guanine nucleotide exchange factor, which activates Ras proteins by exchanging GDP for GTP. The initial binding of Ras-GTP dimers to Raf recruits the RAF/14-3-3 complex to the plasma membrane and relieves the closed conformation by displacing 14-3-3 from the N-terminal binding site. The Ras-induced conformational changes initiate activating modifications such as dephosphorylation of the N-terminal 14-3-3 binding site and phosphorylation of the activation loop, thereby priming Raf for dimerization. The subsequent phosphorylation of the N-region strengthens the Ras/Raf binding and stabilizes Raf side-to-side dimerization, which in turn stabilizes Ras nanoclusters and Ras mediated Raf activation. In the constitutive KSR/MEK complex the inaccessible activation segment of MEK is released through the side-to-side interaction of KSR with a regulatory Raf molecule, allowing catalytic Raf molecule of the Raf:Raf dimer to phosphorylate MEK in trans (RD, regulatory domain; KD, kinase domain; NR, N-region; AS, activation segment). (B) Role of G-proteins in Raf dimerization. The GTPases Rheb and DiRas3 inhibit Raf dimer formation and antagonize Ras-induced Raf activation through different mechanisms. DiRas3 forms heterodimers with activated Ras, thereby disturbing the stoichiometric formation of oligomeric Ras:Ras/Raf:Raf complexes in the nanoclusters and subsequently impairing Raf dimerization and activity. Rheb-GTP forms a complex with Ras/Raf and inhibits phosphorylation of the N-region, thereby destabilizing Ras/Raf interaction and inhibiting Raf dimerization and activation.

regulation is the same, the mechanism of inhibition is different. DiRas3 is part of the Ras:Raf-1 and Ras:B-Raf complexes and actually stabilises the interaction between the Rafs and activated H-Ras.¹⁷ However, DiRas3 specifically binds to activated H-Ras, but not to the RBD domain of Raf.¹⁸ Thus, DiRas3 may interfere with Ras dimerization thereby disturbing the stoichiometric

formation of oligomeric Ras:Ras/Raf:Raf complexes in the nanoclusters and subsequently impair Raf dimerization and activity (Fig. 1B). Surprisingly, DiRas3 does not block the phosphorylation of the N-region in Raf-1 or B-Raf¹⁷, and selectively impairs Raf-1 activity while B-Raf activation is not affected. The mechanism underlying these differential effects is

currently unclear, but could be related to the different activation mechanisms of Raf-1 and B-Raf. For instance, B-Raf is activated by Ras alone, whereas Raf-1 requires additional activators.¹⁹ Thus, DiRas3 may interfere with the activation cycle downstream of Ras at a point where B-Raf is already active but Raf-1 is not. Alternatively, and not mutually exclusively these results could mean that Raf-1 activation is more dependent on direct physical binding to B-Raf than vice versa. By contrast, activated Rheb-GTP interferes with the Ras/B-Raf association, Raf-1/B-Raf heterodimerization and phosphorylation of the N-region (Fig. 1B).¹⁶ Rheb-GTP inhibits kinase activity of both Raf-1 and B-Raf, but selectively interacts with B-Raf, and not Raf-1, indicating that its regulation of Raf-1 must be exerted via B-Raf.¹⁶ Currently, it remains unclear whether Rheb and DiRas3 interfere with Raf dimerization by direct binding to the dimerization interface of Raf, or indirectly by recruitment of other inhibitors or perturbation of the formation and function of Ras nanoclusters. The orchestration of Raf dimerization by different activating and inhibitory Ras family GTPases confers an additional level of complexity to the signal transduction network that may ensure fine-tuning of signalling intensity, duration and subcellular location.

Interestingly, oncogenic B-Raf mutants that are kinase impaired and rely on Raf-1 to activate the ERK pathway can dimerize with Raf-1 in the cytosol leading to Ras independent activation.¹⁰ Likewise, the Raf kinase domain has an intrinsic propensity to dimerize and activate ERK signalling in a Ras independent manner, and alternative splicing that eliminates a large part of the regulatory domain including the RBD from mutated B-RafV600E is a main mechanism exploited by melanomas to become resistant to pharmacological Raf inhibitor.²⁰ Thus, dimerization seems to be a default property of the Raf kinase domain that is under negative control of the regulatory domain.

Mode and function of Raf dimerization

What is the role of Raf dimerization? Structural studies²¹⁻²³ revealed that Raf proteins dimerize side-to-side with contacts mainly made between the N-terminal lobes of the Raf kinase domain. This interaction involves helix α C positioning it in a conformation competent for catalysis. Helix α C is a key regulatory structural element that participates in the allosteric activation of the EGF receptor and other kinases.²⁴ These structural studies inspired a series of elegant mutational analyses to elaborate the following working model. Raf dimerization leads to an allosteric activation of the binding partners provided that one partner has adopted an activated conformation, which is hallmarked by the formation of a “hydrophobic spine” in the protein structure that can be induced by naturally occurring oncogenic mutations in B-Raf, Raf inhibitor drugs that occupy the ATP binding pocket, or artificial mutations that introduce a bulky residue into the ATP binding pocket.²⁵ Thus, a main role of dimerization seems the induction and stabilization of the activated conformation.

This view is supported by findings that KSR, a scaffold protein for the Raf-MEK-ERK pathway, also interacts with Raf by a

similar mechanism causing allosteric Raf activation.^{23, 25} Interestingly, this allosteric activation is mutual and endows KSR with kinase activity towards MEK.^{21, 25} KSR has substitutions in amino acids required for kinase function and hence was widely considered as pseudokinase.⁶ While the allosteric activation by dimerization with Raf kinases may change this view, the substrate specificity of the acquired KSR kinase function needs to be clarified. One group reported that KSR phosphorylates MEK on the activating serines in the activation loop that are also phosphorylated by Raf kinases²⁵, whereas another found that the KSR kinase activity was mainly directed towards novel sites of unknown function in the MEK N-terminus.²¹ However, the latter study also proposed that the formation of a ternary complex between KSR2, MEK1 and Raf re-orientates the MEK1 activation loop to allow phosphorylation by Raf. Notably, this phosphorylation must be carried out by a different Raf molecule, as the Raf bound to KSR2 is sterically unable to phosphorylate MEK and purely serves a structural function that makes MEK a more efficient substrate for Raf (Fig. 1A).

This observation raises an interesting point, namely whether dimerization could aid in substrate recognition and thereby regulate both substrate specificity and catalytic efficiency. Raf-1:B-Raf dimers are much better MEK kinases than the respective monomers or homodimers.¹¹ By analogy with the KSR2:MEK1:Raf complex a Raf heterodimer may enhance MEK phosphorylation by one protomer binding and inducing a MEK conformation that is favourable for phosphorylation by the other protomer. B-Raf binds MEK stronger than Raf-1²⁶, while Raf-1 is the weaker kinase¹⁹. Hence, in the context of the heterodimer B-Raf may present MEK to Raf-1 in a conformation amenable for phosphorylation. Combined with an allosteric activation of Raf-1 by B-Raf, such a double allosteric coercion into activation may explain the high kinase activity of the Raf heterodimer. However, this hypothesis leaves the puzzle why evolution has added two rather poor MEK kinases, Raf-1 and A-Raf, to the ancestral B-Raf, which has good activity. Could this have been to expand the range of substrates beyond MEK? Evidence is still sketchy, albeit several alternative Raf substrate candidates have been identified²⁷ including, for example, adenylyl cyclases²⁸, the retinoblastoma tumour suppressor protein²⁹, the pro-apoptotic BAD protein³⁰, and the eukaryotic translation elongation factor 1A.³¹ In addition, Raf-1 and B-Raf enhance the ability of protein kinase C- θ to phosphorylate BAD, presumably by serving as scaffolds that facilitate substrate binding.³² Kinases are genuinely promiscuous and achieving substrate specificity requires an act of collaborative persuasion where several mechanisms cooperate.³³ Using scaffolding and docking interactions for selective substrate binding seems an effective and versatile mechanism to direct kinases to their substrates. If this selection also involves the activation of kinase function by allosteric mechanisms or other regulatory events, specificity becomes wedded to catalytic efficiency. This dual mode of regulation seems to apply to Raf dimers, but may constitute a more general mechanism to lend specificity and efficiency to phosphorylation dependent signalling.

There are more subtleties that support such a concept. Meticulous mutational analysis of the main dimerization interface

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Fig. 2 Raf dimerization induced by Raf inhibitors. In tumors driven by oncogenic Ras mutations, Raf inhibitors induce increased B-Raf:Raf-1 dimerization resulting in enhanced activation of MEK and ERK. Raf inhibitors also induce robust association of KSR with B-Raf, which in contrast to inhibitor-induced B-Raf:Raf-1 heterodimerization is not dependent on activated Ras. Therefore, in tumours driven by B-RafV600E, treatment with Raf inhibitors induces preferential binding of B-RafV600E to KSR and attenuates the activating effects of Raf inhibitors on MEK and ERK activation.

(DIF) in the N-terminal lobe showed that the abilities to dimerize and transactivate can be separated^{34, 35}, and that an intact DIF is required for the transactivation of wild-type B-Raf and Raf-1 in the context of the dimer. Interestingly, DIF mutations also affected the subcellular distribution enhancing the localisation of A-Raf and Raf-1, but not B-Raf proteins at the cell membrane.³⁴ A further role of the DIF is the binding of phosphatidic acid (PA)^{36, 37}, which promotes membrane localisation and activation by Ras.³⁸ The role of PA in Raf dimerization is unexplored, but PA participates in the assembly of mTOR kinase complexes.³⁹ Thus, by analogy PA may either facilitate Raf dimerization or preferentially bind to the dimer, which may contribute to the propensity of Raf dimers to locate to the membrane.^{22, 40} Furthermore, all structural studies were carried out on isolated Raf kinase domains lacking the regulatory N-terminal parts, and it is unclear whether these findings can be seamlessly extrapolated to the full-length proteins. Fine mapping of the interaction sites in full length Raf-1 and B-Raf proteins using peptide arrays revealed a rather complex mode of interactions involving multiple contact sites within the N-terminal regulatory part as well as within the kinase domain of Raf proteins.¹¹ Interestingly, the results suggest that the interaction interfaces are slightly different for Raf-1:B-Raf heterodimers and the respective homodimers, which may provide a structural basis for the higher

kinase activity of the heterodimer versus the homodimers. These mapping results are supported by structural studies showing that the interaction interfaces differ between KSR2:B-Raf heterodimers and the respective homodimers.²¹ So, what does all this mean?

The pathophysiology of Raf dimerization

Raf dimerization plays a crucial role in diseases associated with cell proliferative disorders. In tumours the most prevalent B-Raf mutation is V600E, which strongly enhances kinase activity as it introduces a phosphomimetic negative charge into the activation loop and disrupts its interaction between the P-loop shifting the kinase domain into the active conformation.⁴¹ A small number of tumour associated B-Raf mutations have impaired MEK kinase activity in vitro, yet they still activate MEK and ERK in cells.^{10, 40, 41} Similar kinase impaired mutations of Raf-1 affecting residues within the activation segment of the kinase domain were found in Noonan syndrome.^{42, 43} Likewise, these Raf-1 mutations paradoxically activate MEK and ERK in vivo.⁴⁴ A mechanism behind this contradictory phenomenon was first suggested by Wan et al.⁴¹ and later confirmed in several other studies.^{10, 11, 40, 44} These Raf mutants cannot directly phosphorylate MEK, but they activate endogenous wild-type Raf

proteins by heterodimerization. Indeed, kinase-impaired B-Raf and Raf-1 mutants possess significantly increased heterodimerization ability compared to wild-type Raf proteins.^{10, 40, 41, 44}

Remarkably, similar to kinase inhibiting mutations many Raf inhibitors enhance the dimerization of Raf kinases, and the binding of such a drug to one Raf protomer in the dimer transactivates the other protomer (Fig. 2).^{22, 40, 45, 46} The result is a paradoxical activation of the ERK pathway, which is thought to cause some of the clinical side effects of Raf inhibitors including a high frequency of keratoacanthomas and squamous cell carcinomas.⁴⁷ These skin tumours are malignant, but curable by surgical excision. Naturally, this side effect causes concern as it is yet unknown whether it also can affect other organs. Understanding Raf dimerization may hold the key to solve this problem, and this quest has turned the spotlights on Raf dimers.

How do drugs and mutations induce Raf dimerization? Both utilize the side-to-side interaction^{22, 44, 46}, which brings the α C helix of the kinase domain into a productive conformation.⁴¹ Interestingly, all currently used Raf inhibitors target the ATP binding pocket, but can stabilize either the kinase active or inactive conformation. Drugs stabilizing the inactive conformation can be further divided into two sub-classes depending on the configuration of the α C helix.⁴⁸ Interestingly, all three classes of Raf inhibitors induce Raf dimerization and activate the ERK pathway in cells, indicating that inhibition of the kinase itself is crucial for the stabilization of Raf dimers rather than conformational changes characteristic for the active or inactive form. This hypothesis is supported by the finding that the 'classical kinase dead' B-Raf mutant (K483M) is still capable to activate MEK in cells.⁴⁰ The highly conserved lysine 483 chelates the α and β phosphate groups of ATP and is essential for orienting ATP for catalysis. The study of similar mutants of PKA (cAMP-dependent protein kinase)⁴⁹ and ERK2⁵⁰ revealed that although phosphoryl transfer is abolished, the mutants are capable of binding ATP, suggesting that the ATP binding ability of the B-Raf(K483M) mutant is intact. Thus, the common feature of the 'classic kinase dead' Raf and the inhibitor-bound Raf is that the ATP binding pocket is occupied by ATP or an ATP analogue, which cannot be hydrolyzed and released. Of note, studies on PKA also showed that occupancy of the ATP binding pocket by ATP or drug increases the structural stability of the catalytic subunit.⁴⁹ Thus, the common denominator of the mutation and inhibitor induced Raf dimerization is the prolonged occupancy of the ATP binding pocket, which stabilizes the dimerization-promoting conformation. This predicts that the efficiency of dimerization is determined by the on/off-rate ratio of the drug or ATP. In fact, all ATP-competitive Raf inhibitors tested so far efficiently induce B-Raf:Raf-1 heterodimerization with the notable exception of PLX4720 and its clinically used relative PLX4032 (vemurafenib).^{22, 40, 46} Currently, it is unclear why the induction of B-Raf:Raf-1 heterodimerization by the PLX compounds is inefficient. Structural studies revealed that binding of PLX4720 to B-Raf induces a shift in the α C helix, bringing it closer to the dimer interface, which may affect dimerization with Raf-1.⁵¹ However, this assumption awaits experimental confirmation. Interestingly, the poor ability of PLX4720 to induce B-Raf:Raf-1 dimerization is vastly enhanced by treatment with the MEK inhibitor PD184352⁴⁰, suggesting that ERK

mediated feedback phosphorylation of B-Raf^{11, 52} or Raf-1^{53, 54} may be involved, but current data do not propose a coherent hypothesis.

Raf dimerization and drug resistance

Due to the spectacular success of vemurafenib in the treatment of melanoma Raf kinase inhibitors have delivered a breakthrough for cancer treatment comparable to the success of imatinib for the therapy of chronic myelogenous leukaemia (CML). A striking observation was that vemurafenib gives high response rates for the treatment of melanomas with B-Raf mutations, but is ineffective against tumours with activating Ras mutations where it even can promote tumour growth.⁵⁵ Moreover, drug resistance develops within months.⁵⁵ The mechanisms are plentiful. Most of them bypass the blockade of B-Raf, but a notable exception is the expression of an alternative B-Raf splice form that can readily dimerize.⁵⁵ This finding and the observation that mutant K-Ras and N-Ras promote Raf dimerization¹³ highlight Raf dimerization as a source for both drug resistance and unwanted side effects. The ramifications are even wider.

Imatinib, nilotinib and dasatinib, inhibitors of the BCR-ABL tyrosine kinase, which is an oncogenic fusion protein and the pathogenetic principle that drives CML, also cause paradoxical activation of Raf.⁴⁵ Although the off-target activity of these drugs against Raf is weak, they efficiently bind to Raf in tumours that have acquired point mutations which block drug binding to BCR-ABL. Due to the inhibitor resistance of the mutated BCR-ABL, Ras activity persists during the treatment with drug, and consequently, paradoxical activation of Raf can be induced by inhibitor. Remarkably, co-treatment with MEK inhibitor suppresses proliferation and induces apoptosis, revealing that CML cells containing drug-resistant mutant of BCR-ABL develop an unexpected dependence on MEK signalling.⁴⁵ Currently, it is not completely understood how inhibition of MEK induces apoptosis under these conditions. A plausible possibility is that these effects are due to the negative feedback amplifier structure of the Raf-MEK-ERK pathway, which predicts and explains a synergism between Raf and MEK inhibitors based on the inherent functional properties emerging from such a design.⁵⁴ An alternative, but not mutually exclusive possibility arises from findings that MEK inhibitors can modulate Raf heterodimerization^{11, 52}, which might prevent Raf-1 from binding to and inhibiting proapoptotic targets. A series of previous studies revealed that Raf-1 can suppress apoptosis in an ERK-independent manner by sequestering several proapoptotic targets including ASK1⁵⁶, Rok- α ⁵⁷ and MST2⁵⁸. Interestingly, in all cases the inhibition of apoptosis did not require Raf-1 kinase activity, but just binding to these targets. In fact, the potency to inhibit the pro-apoptotic MST2 kinase seems to be inversely related to Raf kinase activity. The best MST2 inhibitor is A-Raf, which has barely measurable activity to phosphorylate MEK, followed by Raf-1 and B-Raf, which is the most effective MEK kinase, but ineffective in regulating MST2.^{59, 60} This pattern raises the intriguing question whether Raf inhibitors also impinge on the kinase-activity independent regulation of apoptotic pathways by Raf-1 and A-Raf. Additionally, an increased amount of Raf dimers could lead to increased competition with PI3K for

binding to Ras nanoclusters resulting in suppression of anti-apoptotic PI3K-Akt signalling.¹³ This mechanism may also explain the recently reported dramatic response of NSCLC (non-small cell lung cancer) patients with the kinase-inactivating B-Raf mutation B-Raf(Y472C) to dasatinib treatment. This B-Raf mutation enhances B-Raf:Raf-1 heterodimerization, which in cooperation with dasatinib treatment induces apoptosis in cancer cells.⁶¹

Raf inhibitors also induce robust association of KSR1 or KSR2 with B-Raf, which in contrast to inhibitor-induced B-Raf:Raf-1 heterodimerization is not dependent on activated Ras.⁶² Furthermore, in tumours driven by B-RafV600E treatment with Raf inhibitors induces preferential binding of B-RafV600E to KSR1, but little B-RafV600E:Raf-1 heterodimerization. These results suggest that KSR1 competes with Raf-1 for drug-induced binding to B-RafV600E in tumours with low Ras activity and attenuates the activating effects of Raf inhibitors on ERK activation (Fig. 2). These results suggest that KSR levels may modulate the sensitivity to B-Raf inhibitors. This possibility has not been directly tested yet, but vemurafenib treatment of drug resistant A375 melanoma cells (which express B-RafV600E) elevated the concentrations of KSR1 and Raf-1 proteins, and enhanced ERK activation.⁶³ Unfortunately, the dimerization status of KSR1, B-Raf and Raf-1 was not determined in this study, but based on the KSR studies described above^{25, 62} expression changes should influence which dimers are formed, suggesting that KSR may play a role in the resistance to Raf inhibitors.

Conclusion and Outlook

Amongst the protein interactions that have made headlines the Raf dimer is arguably a celebrity. From first reports that chemical Raf inhibitors cause a paradoxical activation of the ERK pathway⁶⁴ more than a decade elapsed before blame was laid on the Raf-1:B-Raf heterodimer.^{22, 40, 45, 46} Since then research on Raf dimerization progressed at breakneck speed driven by the enormous clinical potential of Raf inhibitors and the complications arising from Raf dimerization. Taking together current evidence, some of which is discussed above, suggests that understanding Raf dimerization holds the key to understand and overcome these complications. However, many questions remain.

A pertinent question is the mechanism of the transactivation between Raf protomers. First, there are discrepant findings whether activation is bidirectional where either Raf isoform can activate the other^{11, 46}, or unidirectional where only B-Raf activates Raf-1.^{10, 25} Resolving this issue is important as the unidirectional mode predicts that Raf-1 or pan-Raf inhibitors should be more potent than B-Raf specific inhibitors. The fact that the rather non-selective Raf inhibitor sorafenib causes paradoxical ERK activation similar to B-Raf selective inhibitors, such as vemurafenib, may indicate that activation is bidirectional. An even bigger puzzle is the phenomenon that both Raf inhibitors that bind to the inactive and those which bind to the active conformation induce dimerization and transactivation of Raf, and that there is no clear correlation between the potencies to induce Raf dimerization and activation of the ERK pathway. Thus, the mechanism of transactivation cannot be purely allosteric. A

possibility is that induction of Raf dimerization is only one effect of Raf inhibitors, and that they in addition disrupt the interaction of Raf with a yet unknown inhibitor or enhance the interaction with a co-activator. A candidate for the latter is KSR²⁵ or A-Raf, which was recently discovered to act as scaffold that stabilizes Raf-1:B-Raf heterodimers.⁶⁵

Another question is how Raf dimers affect other pathways controlled by Raf-1. The regulation of ASK1⁵⁶, and ROK- α ⁵⁷ by Raf-1, and MST2 by Raf-1⁵⁸ and A-Raf⁶⁰ does not require Raf kinase activity but is entirely dependent on the ability of Raf-1 and A-Raf to associate with these targets. It is conceivable that Raf dimerization could modulate these interactions positively or negatively, or that Raf inhibitors affect the ability of Raf-1 and A-Raf to interact with these protein kinases. ASK1, MST2 and ROK- α share the ability to induce apoptosis, MST2 and ROK- α also control proliferation and migration, while ASK1 is intimately involved in oxidative stress signalling.⁶⁶ All these traits are clearly relevant for tumour pathophysiology, and alteration of such interactions by Raf inhibitors may contribute to their effects. A related question is whether Raf dimerization affects substrate choice. Raf heterodimers have increased kinase activity towards MEK¹¹, but looking at the other side of the coin may suggest that Raf monomers could have high kinase activity towards other yet unknown substrates. An interesting paradigm is the Toll-like Receptor 4 (TLR4), where the dimer can adopt different conformations when localized at the cell membrane and after internalisation in endosomes, and where these dimeric conformational isoforms can associate with different adaptors to activate different downstream signalling pathways.⁶⁷

Finally, the biggest question of all is, of course, how we can deal with all the problems for cancer therapy imposed by Raf dimerization? The obvious answer seems to be to develop Raf inhibitors that do not induce dimerization or even develop dimerization inhibitors. However, given the poor correlation between the abilities to induce Raf dimerization and paradoxical activation of ERK this head-on charge may not work. A more subtle approach could be to exploit inherent design properties of the ERK pathway as negative feedback amplifier.⁵⁴ The three-tiered kinase cascade Raf-MEK-ERK functions as amplifier module where the output is connected to the input via a negative feedback from ERK to Raf-1. This configuration confers robustness against perturbations of the amplifier, such as MEK inhibitors, and may be the reason why MEK inhibitors, despite being potent and selective, enjoyed limited success in the clinic.⁶⁸ Mathematical modelling supported by experimental evidence showed that attenuating the negative feedback strength by a Raf inhibitor vastly increases the sensitivity to MEK inhibitors.⁵⁴ This prediction of, by conventional wisdom also paradoxical, synergism between Raf and MEK inhibitors is currently explored in clinical studies.⁶⁸ Similarly, playing on the theme of exploiting network connections for circumventing clinical problems arising from Raf dimerization the solutions may lead to solutions that lie further afield. For instance, the Src family tyrosine kinase inhibitor dasatinib induces association of Raf-1 with KSR1, ERK and the tyrosine kinase Lyn, but in this case enhancing the benefit of ERK dependent differentiation of retinoic treated leukaemia cells.⁶⁹

Thus, the simple Raf dimer has posed complex intellectual

challenges that will equally stimulate basic and applied research for some time to come.

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Notes

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