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Raf Family Kinases: Old Dogs Have Learned New Tricks

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Abstract

First identified in the early 1980s as retroviral oncogenes, the Raf proteins have been the objects of intense research. The discoveries 10 years later that the Raf family members (Raf-1, B-Raf, and A-Raf) are *bona fide* Ras effectors and upstream activators of the ubiquitous ERK pathway increased the interest in these proteins primarily because of the central role that this cascade plays in cancer development. The important role of Raf in cancer was corroborated in 2002 with the discovery of B-Raf genetic mutations in a large number of tumors. This led to intensified drug development efforts to target Raf signaling in cancer. This work yielded not only recent clinical successes but also surprising insights into the regulation of Raf proteins by homodimerization and heterodimerization. Surprising insights also came from the hunt for new Raf targets. Although MEK remains the only widely accepted Raf substrate, new kinase-independent roles for Raf proteins have emerged. These include the regulation of apoptosis by suppressing the activity of the proapoptotic kinases, ASK1 and MST2, and the regulation of cell motility and differentiation by controlling the activity of Rok-α. In this review, we discuss the regulation of Raf proteins and their role in cancer, with special focus on the interacting proteins that modulate Raf signaling. We also describe the new pathways controlled by Raf proteins and summarize the successes and failures in the development of efficient anticancer therapies targeting Raf. Finally, we also argue for the necessity of more systemic approaches to obtain a better understanding of how the Ras-Raf signaling network generates biological specificity.

Keywords: Raf kinases, signal transduction, cancer, apoptosis, kinase inhibitors

Introduction

The first *raf* (rapidly accelerated fibrosarcoma) gene was described in 1983 as a retroviral oncogene, *v-raf*, transduced by the murine sarcoma virus isolate 3611.[1] A year later, an avian homolog, *v-mil*, was found in the MH2 retrovirus.[2] These 2 transforming retroviruses encoded the first oncogene to be discovered with serine/threonine kinase activity.[3] After the cellular proto-oncogene homologs, *c-raf*[4] and *c-mil*,[5] had been cloned, studies focused on elucidating the function of Raf proteins. They showed that c-Raf (also known as Raf-1) plays a critical role in mediating the cellular effects of growth factor signals.[6-8] Later on, Raf proteins were identified as direct activators of MEK[9,10] and effectors of Ras.[11-15] Thus, Raf proteins were placed as essential connectors between Ras and the MEK-ERK pathway (Fig. 1). Most subsequent work focused on understanding this role and the regulation of Raf proteins in detail, until new functions of Raf-1 in the regulation of apoptosis[16-18] and cell migration[19] emerged in the last decade.

Three different Raf isoforms originating from 3 independent genes can be distinguished in mammals, Raf-1/c-Raf, B-Raf, and A-Raf. Raf-1 was the first isoform to be identified[4] and for 20 years was the principal focus of attention on the proteins of the family. After the discovery 8 years ago of B-Raf mutations in different types of tumors,[20] B-Raf moved into the limelight, resulting in a rapid increase of our knowledge of the biological functions of this isoform. On the other hand, still very little is known about A-Raf, and although it seems to share many of the properties of the other isoforms, its biological functions remain a mystery. All Raf proteins share MEK1/2 kinases as substrates. MEK1/2 in turn activate ERK1/2, and this pathway regulates many cellular functions such as cell proliferation, differentiation, migration, or apoptosis (for extensive reviews, see Wellbrock *et al*.,[21] Leicht *et al*.,[22] and Dhillon *et al*.[23]).

In recent years, it has become clear that the initial view of the ERK pathway as a linear pathway is not accurate but that there are many different proteins interacting with the proteins of the pathway. These proteins regulate the pathway by mediating the crosstalk with other signaling pathways and the regulation of positive and negative feedback mechanisms.[24] In this review, we focus on the mechanisms of Raf family regulation and the biological roles of Raf family kinases especially in cancer and with relation to Ras signaling. We also explore the role of the Raf proteins in the context of the coordinated signaling networks that ultimately are responsible for cellular responses both in normal and

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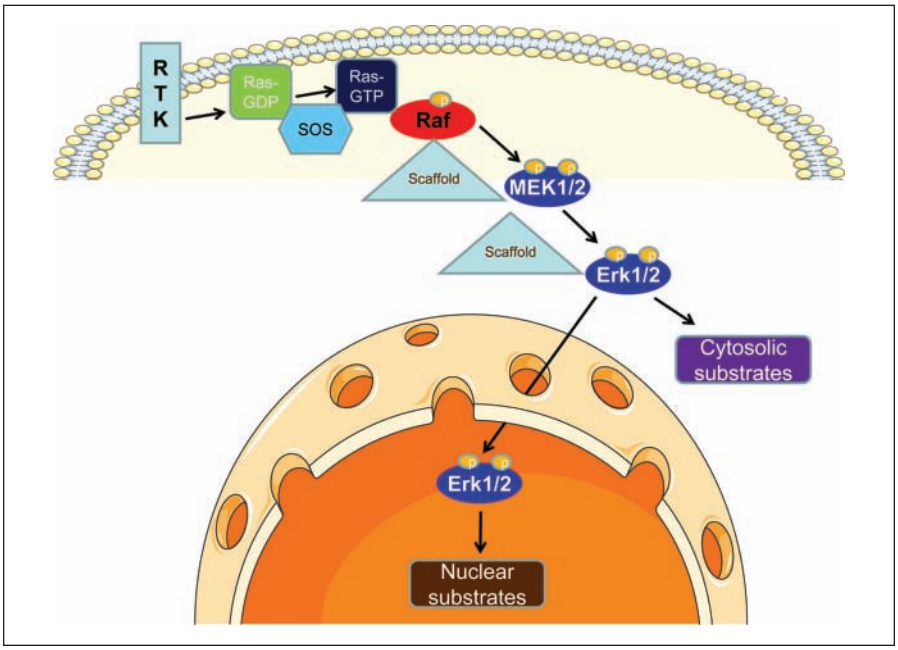

Figure 1. The prototypical Ras-Raf-MEK-ERK pathway. Activated receptor tyrosine kinases (RTKs) recruit the guanine nucleotide exchange factor SOS, which activates Ras proteins by exchanging GDP for GTP. Activated GTP-loaded Ras binds to Raf, initiating Raf activation. Active Raf phosphorylates and activates MEK, which in turn phosphorylates and activates ERK. While the phosphorylation cascade comprising Raf, MEK, and ERK is linear, ERK features more than 150 substrates both in the cytosol and nucleus.[427] Protein interactions and phosphorylation reactions are modulated by a number of scaffolding proteins (see Fig. 5).

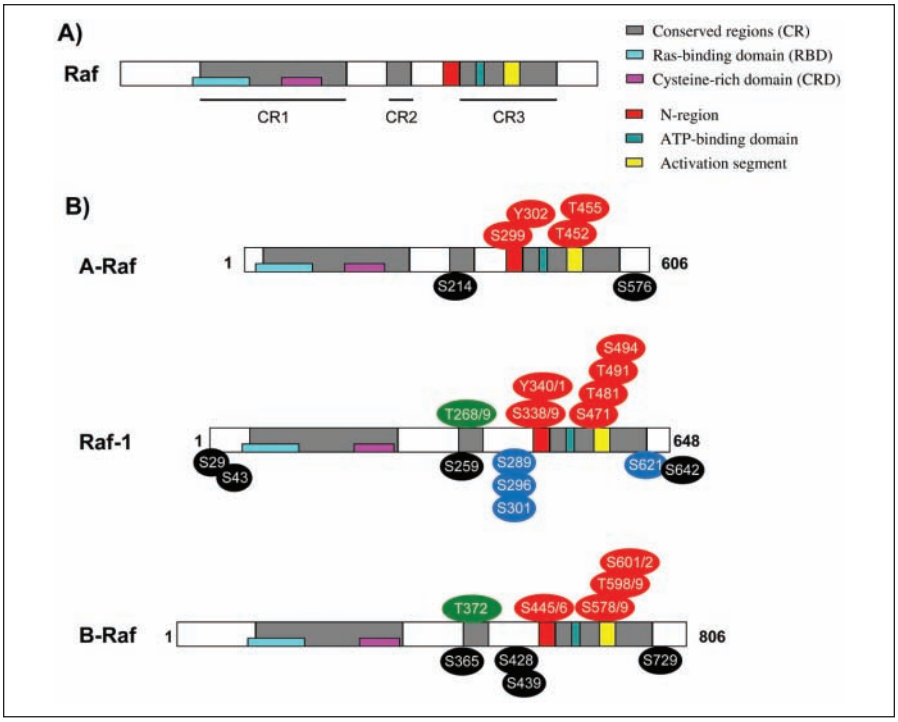

Figure 2. Structure and regulatory phosphorylation sites of Raf proteins. (**A**) Common structure of the Raf proteins. Color-coded regions are described in the text. (**B**) Comparison of the structure and phosphorylation residues of the 3 Raf isoforms. Red residues indicate activating phosphorylation sites, black are inhibitory sites, and blue are sites that have been described as both activating and inhibitory. The major *in vitro* autophosphorylation sites in Raf-1[428] and B-Raf[429] are in green.

tumor cells. We end on how systems biology can help us to integrate the information gathered from the many years of research in the Ras-Raf pathway and how it can be used to address open questions.

Structure and Regulation of Raf Isozymes

Raf Structure

There are no Raf kinases in yeasts, and the phylogenetic oldest isoform is B-Raf, which appears in invertebrates. Mammals possess 3 Raf isoforms (Raf-1, B-Raf, and A-Raf), which share a common modular structure consisting of 3 conserved regions (CR) with distinct functions (Fig. 2). CR1 contains a Ras-binding domain (RBD), which is necessary for the interaction with Ras and with membrane phospholipids required for membrane recruitment, and a cysteine-rich domain (CRD), which is a secondary Ras-binding site and also necessary for the interaction of CR1 with the kinase domain for Raf autoinhibition.[25] CR2 contains important inhibitory phosphorylation sites participating in the negative regulation of Ras binding and Raf activation.[26] CR3 features the kinase domain, including the activation segment, whose phosphorylation is crucial for kinase activation.[27] Unfortunately, the tertiary structure of a Raf holoenzyme has remained elusive, although the structures of the RBD and extended CR1 domains of Raf-1[28-30] and the CR3 domain of B-Raf[31] and Raf-1[32] were solved. Functionally, the Raf structure can be split into a regulatory N-terminal region, containing the RBD, which is critical for activation as well as inhibitory phosphorylation sites, and a catalytic C-terminal region, which includes phosphorylation sites necessary for the kinase activation. The regulatory domain restrains the activity of the kinase domain,[25,33,34] and its removal results in constitutive oncogenic activation.[35] However, the activity of the isolated Raf-1 kinase domain is subjected to further regulation and can be stimulated by phorbol esters, v-Src, and phosphorylation.[34,36] This observation is in keeping with the

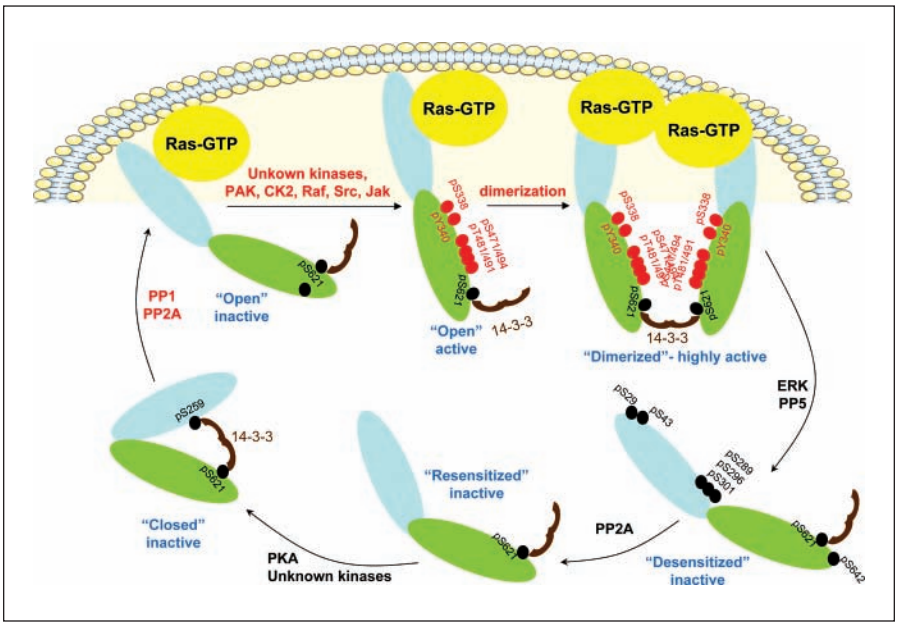

Figure 3. The Raf-1 activation/deactivation cycle. This scheme shows the salient steps in Raf-1 activation/deactivation. Activating events are coded red, inactivating processes are in black, and activation states are in blue. In quiescent cells, Raf-1 is phosphorylated on both 14-3-3 binding sites pS259 and pS621, and 14-3-3 maintains the closed inactive conformation. Upon membrane recruitment by activated Ras, pS259 is dephosphorylated by the corecruited phosphatases PP1 or PP2A. Subsequently, phosphorylation of the N-region and activation loop and homodimerization or heterodimerization (with B-Raf) cause full activation of Raf-1. Deactivation is initiated by pS338 inducing PP5 binding and dephosphorylation of pS338. In addition, ERK-mediated feedback phosphorylation suppresses Raf-1 catalytic activity. Eventually, PP2A (and maybe other unknown phosphatases) dephosphorylates the remainder of activating sites and the ERK feedback sites. Rephosphorylation of S259 allows intramolecular bidentate 14-3-3 rebinding and return to the inactive state.

finding that the most common oncogenic mutation in B-Raf, V600E, activates B-Raf kinase activity by mimicking phosphorylation of the activation loop that releases its inhibitory interaction with the ATP-binding domain.[31]

The Raf-1 Activation Cycle

In the inactive state, Raf-1 is thought to exist in a closed conformation in which the N-terminal regulatory region folds over and occludes the catalytic region.[37] This conformation is stabilized by a 14-3-3 dimer binding to an N-terminal site, phospho-S259 (pS259), and a C-terminal site, pS621. Although the activation process of Raf-1 is not completely understood, we can assume the following sequence of events (Fig. 3).

1. Dephosphorylation of pS259 at the cell membrane by specific phosphatases (PP2A, PP1) releases 14-3-3 from its N-terminal binding site in Raf-1, thereby allowing conformational changes to occur that unmask the RBD and CRD domains in the CR1 region to enable Ras binding and membrane recruitment.[38-44]
2. Ras binding itself has several intricate facets. The RBD is essential for the selective binding to activated RasGTP, and this binding interface was extensively characterized structurally[28,29] and functionally.[45] A notable observation was that in the absence of feedback, the Ras–Raf-1 association rate kinetics rather than the total affinity determined the extent of downstream ERK activation. However, negative feedback operating from ERK back to Ras activation negated the subtlety of the kinetic effects and rendered ERK activation transient.[45] This negative feedback is mediated by ERK and its downstream substrate RSK terminating Ras activation by phosphorylating and inhibiting the guanine exchange factor SOS.[46,47] In this context, it is interesting that the binding of Raf-1 to Ras can be accelerated by the scaffolding protein Sur-8/SHOC2.[48] A complex between Sur-8/SHOC2 and the catalytic subunit of PP1c is an effector of the Ras family protein M-Ras, which can dephosphorylate the inhibitory pS259 in Raf-1 at the membrane.[49] Thus, Ras proteins can cooperate in recruiting Raf-1 and specific activators. The CRD exhibits constitutive low affinity for Ras that does not discriminate between Ras activation states.[50] The CRD is not sufficient but necessary for stable membrane recruitment and activation of Raf-1.[51,52] These results suggest that the CRD may stabilize the primary recruitment of Raf-1 exerted by the RBD through forging interactions with the farnesyl lipid tails of Ras proteins.[50,53] In addition, Raf translocation to the membrane is aided by the ability of all 3 Raf isoforms to interact with lipids.[54-57] In fact, as phosphatidic acid (PA) can bind to both Raf-1[54] and SOS,[58] PA was suggested to nucleate Ras nanocluster formation in response to EGF.[59] Furthermore, Ras isoforms reside in different microcompartments, which can influence interactions with Raf kinases.[60,61] This spatial organization may profoundly influence the mechanism and kinetics of Raf activation by different Ras isoforms.[60] Ras itself seems to be organized in short-lived, highly dynamic nanoclusters, which activate Raf-1 in a digital way; that is, each Ras nanocluster produces a constant output of Raf activity. However, as the number of Ras nanoclusters increases proportionally with growth factor concentrations, the overall output is analog.[62] This conversion of an analog input signal into an analog output by means of a digital intermediate increases the fidelity of signal transmission across the cell membrane. Interestingly, Ras isoforms also can reside at and signal from endomembranes including the Golgi apparatus and endoplasmic

reticulum.[60,63,64] The subcellular localization modulates the efficiency with which different effector pathways are engaged but, in the case of Raf-1, also has a major influence on the dynamics of signaling. While increasing doses of EGF induced ERK activation in a linear fashion when signaling from the plasma membrane early after EGF treatment, the dose response became nonlinear and sigmoid when activated from the Golgi at later time points.[60] Thus, according to this model, different EGF concentrations are translated into ERK activity in a highly linear, quantitative fashion at the cell membrane in the early phase of signaling. At later time points, when ERK is activated from the Golgi, the dose response curve becomes nonlinear with a less accurate input-output relationship. The physiological role of these dynamic differences was demonstrated in T cell selection, where strong ERK activation at the plasma membrane drove negative selection, whereas delayed signaling from the Golgi promoted positive selection.[65]

3. Phosphorylation of the activation segment in CR3 and the "N-region" (negative charge regulatory region) is upstream of CR3. The N-region contains the $S^{338}SYY^{341}$ phosphorylation sites, which are not only essential for full kinase activation but also for interaction with the substrate MEK.[66-68] The kinases, which can phosphorylate Y341, include Src6[9,70] and JAK family kinases.[71] Mutation of Y341 severely compromises Raf-1 kinase activity,[69,70] but detecting phosphorylation of this residue in response to physiological stimuli is difficult, and most studies used overexpressed or mutated tyrosine kinases. This observation may indicate that our current detection methods are insufficient or that the modification is instable or actually a different chemical entity that provides a negative charge such as a sulfate group. Phosphorylation of S338 is detectable reliably and used routinely as a surrogate marker for Raf-1 activation. Pak family kinases were reported to phosphorylate S338 in response to growth factor stimulation[72,73] and integrin activation.[74] However, the role of Pak in Raf-1 activation was questioned because stimuli that activate Raf-1 did not necessarily activate Pak and because Pak phosphorylation did not automatically activate Raf-1.[75] More recent work suggested that S338 could be an autophosphorylation site induced by dimerization[76] or be a target for casein kinase 2 (CK2) recruited to Raf-1 and B-Raf by the scaffold KSR.[77] Given that Raf kinases can be activated by many diverse stimuli, it is not surprising that key phosphorylation sites are targeted by several different kinases. Interestingly, S338 phosphorylation is not required for Raf-1 activation at the Golgi,[60] suggesting that the activation mechanism at the Golgi membrane may be different from the activation mechanism at the plasma membrane. S338 phosphorylation itself only slightly elevates Raf-1 kinase activity[66] and mainly seems to serve as a priming event that initiates further activating modifications. Both Ras and Raf-1 activation at the Golgi is delayed relative to the plasma membrane,[60,65] indicating that the initial Raf-1 priming events may be different between these compartments. Alternatively, recent evidence suggests that H-Ras is activated at the plasma membrane and endoplasmic reticulum and then delivered to the Golgi.[78] In this scenario, Raf-1 could be activated at the plasma membrane and travel to the Golgi bound to activated H-Ras. As pS338 rapidly recruits the protein phosphatase PP5 to Raf-1,[79] this residue may become dephosphorylated during this journey. However, activity may be maintained, as in this state, Raf-1 already would have undergone dimerization with B-Raf or KSR, which can activate Raf-1 allosterically.[80-82] At the Golgi, Raf-1 and B-Raf can associate with the Raf kinase trapping to the Golgi (RKTG) protein, which inhibits Raf signaling by interfering with Raf binding to Ras and MEK.[83,84] The existence of such Golgi-specific Raf regulatory proteins suggests that the Golgi may have developed its own means to regulate Raf activity. Finally, the phosphorylation of 2 sites in the activation loop is required for full activation[85,86] and activation by Raf heterodimerization,[81] but the identity of the respective kinases is unknown. In addition, S508 in the Raf-1 activation loop is involved in MEK binding.[87]
4. Raf homodimerization and heterodimerization recently emerged as important regulatory mechanisms to drastically enhance the kinase activity and signaling of Raf. It is not entirely clear at which step in the activation dimerization occurs. Although it is part of the physiological activation mechanism,[82] it may also provide an alternative route of Raf activation independent of N-terminal phosphorylation.[81] Because of the mechanistic complexity and relevance for mutant Raf signaling and drug responsiveness, it is discussed in detail below.
5. Deactivation is initiated by specific binding of PP5 to activated Raf-1, which results in the dephosphorylation of pS338, rephosphorylation of S259, and return into the inactive state.[79] The phosphorylated N-region also serves as a binding site for the Raf kinase inhibitor protein (RKIP),[88,89] which dissociates Raf-1 from its substrate MEK.[90,91] In addition, Raf-1 is subjected to direct feedback phosphorylation by ERK on 6 sites, which inhibits the activation of Raf-1 by Ras and promotes the subsequent dephosphorylation of Raf-1 by PP2A and the return to the inactive state.[92] A negative feedback from ERK to Raf-1 was also confirmed by a systematic analysis of feedback regulation of the ERK pathway based on mathematical modeling.[93] However, ERK feedback

phosphorylation also was described as stimulating Raf-1 activity.[94] The reason for this contradiction is not clear. While Dougherty *et al*.[92] reported 6 feedback sites, Balan *et al*.[94] identified a subset of 3 of the 6 sites. Two of these 3 sites were also identified as stimulatory phosphorylation sites in A-Raf,[95] raising the interesting possibility that the phosphorylation of a subset of ERK feedback sites has a positive effect, whereas phosphorylation of the full complement is inhibitory. Such a mechanism could be a simple way to dynamically regulate strength and duration of ERK signaling, where early incomplete phosphorylation would boost Raf-1 activity, while later complete phosphorylation would switch Raf-1 activation off. Another important negative regulation of Raf-1 is phosphorylation by cyclic AMP–activated kinase (PKA). This topic was recently reviewed[96] and therefore is only presented briefly. Raf-1 is a direct PKA substrate, and different studies found several sites in which PKA can phosphorylate Raf-1. The phosphorylation of S43 interferes with binding to Ras,[97,98] while phosphorylation of S233 and S259 enhances the binding of 14-3-3 and suppresses catalytic activity.[99,100] The phosphorylation of S621 has a dual role. It decreases Raf-1 kinase activity,[99,101] but its inhibitory function is converted into an essential component of Raf-1 activity by 14-3-3 binding.[102] S259 also was reported to be phosphorylated by Akt,[103] but this observation could not be reproduced.[104,105]

A-Raf Regulation

A-Raf is generally thought to be regulated similarly to Raf-1, but important differences have emerged. A-Raf is only weakly activated by oncogenic H-Ras and Src and also displays low kinase activity towards MEK.[106] The reason for the lower responsiveness to H-Ras is the exchange of an arginine for a lysine at position 22 in the A-Raf RBD, which weakens the binding of A-Raf to H-Ras.[107] In addition, the low kinase activity may be unique nonconserved amino acid residues in the N-region. Mutation of Y296 in the N-region led to a constitutively active kinase, and molecular modeling showed that Y296 promotes a tighter interaction between the N-region and the catalytic domain, which may stabilize the closed conformation.[108] Subsequently, a systematic phosphorylation site analysis revealed several interesting findings[95]: S432 located between the ATP-binding domain and activation loop was found critical for MEK binding and A-Raf signaling. Surprisingly, activation loop phosphorylation did not contribute to mitogen-induced activation. Finally, a cluster of phosphorylation sites between amino acids 248 and 267, which stimulated activation, facilitated A-Raf dissociation from the plasma membrane. These findings raise some intriguing aspects. First, activation loop phosphorylation, which is a widespread mechanism in the catalytic activation of kinases,[109] may be less critical in Raf regulation, as corresponding structural reorganizations may be caused by 14-3-3 proteins binding to the Raf kinase domain (see below). Second, the fact that activating phosphorylation events release A-Raf from the plasma membrane suggests that while initial activation may occur at the membrane, downstream signaling proceeds in other subcellular compartments. In this context, both A-Raf and Raf-1 have been found in different subcellular compartments including mitochondria, endosomes, and the Golgi apparatus.[110] It is unknown whether A-Raf is regulated by PKA.

B-Raf Regulation

B-Raf activation appears much simpler. In fact, Ras and 14-3-3 binding are likely to be the only major requirement for B-Raf activation.[106,111] The N-region is already negatively charged because of the presence of aspartate at the position corresponding to Raf-1's YY340/1 (DD448/9) and the constitutive phosphorylation of S446 (corresponding to the S338 of Raf-1). Phosphorylated S446 neutralizes the inhibitory role of N-terminal domain towards the catalytic region and in conjunction with D449 allows the catalytic domain to adopt a stabilized 3-dimensional conformation.[25] Phosphorylation of S365 (corresponding to S259 in Raf-1) impairs B-Raf activity, and its mutation to a nonphosphorylatable residue can even overcome the debilitating effects of charge-neutralizing mutations in the N-region.[112,113] In contrast to Raf-1 S259, B-Raf S365 is unlikely to be phosphorylated in cells upon cAMP stimulation, but another site, S429, is a potential target for PKA.[114] Importantly, B-Raf can be inhibited or activated by PKA depending on the levels of 14-3-3 expression, which need to be high for permitting activation.[115] A second pathway may involve the PKA-mediated activation of Rap1, which was reported to bind and activate B-Raf.[116,117] However, these results are disputed, as many physiological signals that induce Rap1 activity fail to activate B-Raf.[118] Possible reasons for this discrepancy are unclear and are discussed below.

The Role of Ras Family Proteins in Raf Isoform Regulation

The common and key step in the activation of all 3 Raf isoforms is membrane recruitment by a Ras family protein. Membrane translocation triggers further activation events, such as the binding of PP2A to dephosphorylate the inhibitory pS259 site in Raf-1 (and presumably the corresponding sites in A-Raf and B-Raf) and the colocalization with the kinases responsible for the multiple activating phosphorylations as discussed above. The sequences forming the binding interface are well conserved in the Raf as well as Ras family. Hence, it is not surprising that several members of the Ras family can bind Raf kinases. A systematic comparison of the ability of different Ras family members to activate Raf isoforms[119] showed that H-Ras, N-Ras, and K-Ras could stimulate all 3

Raf isoforms and were the only Ras proteins that could activate B-Raf. In contrast, A-Raf also could be activated by R-Ras3, while Raf-1 was the most promiscuous isoform, responding also weakly to R-Ras3, Rit, and TC21. In contrast, Rap1/2, Rin, and Rheb were ineffective. The ability to activate Raf generally corresponded with the binding affinity. Only H-Ras, N-Ras, and K-Ras led to a stimulation of the endogenous ERK pathway in HEK293 cells, while lower affinity interactions only could stimulate ERK when either Raf or ERK was overexpressed. These results suggest that cell type–specific expression stoichiometry of Ras isoforms and Raf-ERK pathway components potentially could generate a rich variety of ERK activation dynamics that could allow cells to respond to different growth factors with precisely tuned ERK activation. An example is Rheb, which was reported to interact with Raf-1 and B-Raf and to suppress their kinase activity by reducing N-region phosphorylation and heterodimerization.[120,121] Interestingly, PKA-mediated phosphorylation of Raf-1 on S43 increases Raf-1 affinity for Rheb and thereby could contribute to the inhibitory effects of PKA by diverting Raf-1 from H-Ras to Rheb.[122]

Although this hypothesis is conceptually appealing, the experimental evidence for specific engagement of Raf isoforms by different Ras family members is often controversial. A case in particular is Rap1, which was initially isolated as a repressor of K-Ras transformation.[123] Rap1 binds to Raf-1,[28] but the functional consequences are disputed. Constitutively active Rap1 can inhibit Raf-1 when overexpressed,[124] while at normal expression levels and in response to physiological stimulation, Rap1 did not regulate Raf-1.[118,119] In addition, Rap1 was reported to activate B-Raf[116,117] and mediate cAMP stimulation of ERK and neuronal differentiation of PC12 cells.[117] However, this finding was not reproduced in other studies,[118] and the issue remains open. A possible explanation for the differential effect of Rap1 and Ras on B-Raf and Raf-1 is the difference in affinity to the CRD domains. Rap1 has high affinity for the Raf-1 CRD domain and low affinity for the B-Raf CRD, whereas Ras has low affinity for both Raf-1 and B-Raf CRD domains. Swapping the CRD domains showed that the B-Raf CRD conveyed susceptibility to activation by Rap1, while the Raf-1 CRD abolished it.[116] An alternative, but not mutually exclusive, mechanism could be the failure of Rap1 to target Raf-1 to the membrane compartment, where S338 phosphorylation can take place.[125] Correct targeting needs Y341 phosphorylation or the negative charge that B-Raf carries at this position. Introducing a negatively charged residue at this position or redirecting Rap1 to lipid rafts could overcome the deficiency of Rap1 to activate Raf-1.[125] A recent study using statistical modeling based on Bayesian inference revealed a role for Rap1 in EGF-stimulated ERK activation by supporting Raf-1 and B-Raf heterodimerization.[126] The exact mechanism needs to be elucidated. Apart from direct Rap1 binding to B-Raf, it also could involve indirect effects such as a recruitment of a common scaffold by Rap1. In natural killer lymphocytes, Rap1 was recently shown to bind to the scaffolding protein IQ motif containing GTPase-activating protein 1 (IQGAP1) and to assemble a signaling complex that activated B-Raf, Raf-1, and ERK.[127] Thus, the contradictory findings on Rap1 regulation of Raf proteins may eventually be reconciled when considering indirect mechanisms such as the involvement of scaffolds.

Regulation of Raf Isoform Expression by Differential Splicing

All 3 Raf isoforms are regulated at the level of protein expression. Alternative splicing gives rise to multiple B-Raf isoforms differentially expressed in various tissues.[128,129] B-Raf activity is also regulated by splicing. B-Raf isoforms containing exon 8b are more phosphorylated on the inhibitory S365 site, leading to an increased interaction with 14-3-3 and strengthening the inhibitory interaction between N-terminal regulatory domain and kinase domain, altogether resulting in lower kinase activity.[112] With respect to A-Raf, the 2 splice isoforms described so far, DA-Raf1 and D-Raf2, lack the kinase domain and act as dominant inhibitory mutants of Ras[130] and ARF GTPases.[131] As a consequence, DA-Raf1 is a positive regulator of myogenic differentiation by mediating the inhibition of the ERK pathway required for differentiation.[130] Raf-1 also has a known splice variant preferentially expressed in the muscle and brain.[132]

Raf Regulation by 14-3-3 Proteins

Key regulatory phosphorylation sites in Raf kinases are also binding sites for the scaffolding protein 14-3-3. 14-3-3 is an obligatory dimer forming a rigid half-barrel structure that interacts with other proteins in a phosphorylation-dependent, bidentate way, constraining the conformation of the binding partner.[133] In Raf kinases, 14-3-3 can stabilize both the inactive and the activated state. This dual property confounds the analysis of 14-3-3 effects on Raf kinase regulation and can explain many of the controversies in the literature.

In the inactive configuration, 14-3-3 binds to conserved phosphorylation sites in the N- and C-terminus of the Raf kinase domain (pS259 and pS621 in Raf-1; pS365 and p729 in B-Raf; pS214 and pS576 in A-Raf).[36,134-138] This bidentate interaction is thought to physically clamp the regulatory domain to the kinase domain. Thus, it is not surprising that the 14-3-3 binding sites are targeted by kinases that inhibit Raf activation. Both protein kinase A (PKA) and B (PKB/Akt) were reported to induce phosphorylation of the N-terminal 14-3-3 binding site of Raf-1 and B-Raf.[26,34,98-100,103,114,139-141] However, the relevance of Akt phosphorylation remains disputed, as Akt does not phosphorylate S259 in most physiological scenarios.[104,105] While PKA is a *bona fide* S259 kinase, it is not responsible for the constitutive phosphorylation of S259,[99] and the kinase that maintains

basal S259 phosphorylation in cells is still unknown. The C-terminal 14-3-3 binding residue S621 is targeted by PKA,[101] AMP-activated protein kinase (AMPK),[142] autophosphorylation,[101,102,143] and probably other yet unidentified kinases. The exact role of S621 is still not clear. It was reported to inhibit Raf-1 kinase[101] but also to be essential for kinase activity[137,138] and for the stability of the Raf-1 protein.[143] Recent data[102] show that mutations that preclude 14-3-3 binding to S621 inactivate Raf-1 by specifically disrupting its capacity to bind to ATP rather than by gross conformational alteration, as indicated by the intact ability to bind MEK. Phosphorylation of S621 inhibits Raf-1 catalytic activity *in vitro*, but addition of 14-3-3 proteins completely reverses this inhibition. 14-3-3 binding requires the phosphorylation of S621, and this interaction is essential for Raf-1 kinase activity, but S621 phosphorylation in the absence of 14-3-3 does not support kinase activity. These data explain the dual role of S621 phosphorylation and suggest that 14-3-3 may serve as a switch that can convert an inactive Raf-1 population phosphorylated on S621 into a kinase-competent state.[102] Although B-Raf catalytic activity is less dependent on 14-3-3 binding, its biological activity is dependent on 14-3-3 binding,[144,145] and 14-3-3 binding can switch inhibitory phosphorylation of B-Raf by PKA into activation.[115] An additional role of 14-3-3 in Raf kinase activation is related to its enhancement of Raf-1 homodimerization and Raf-1/B-Raf heterodimerization, which elevates Raf-1 kinase activity[82,146] and is discussed below.

Raf Homodimers and Heterodimers

Dimerization is a common motif in the activation of kinases. Homodimerization was initially highlighted as a potentially important step of Raf-1 activation by 2 studies showing that a forced interaction of Raf-1 monomers tagged with inducible dimerizing tags robustly induced kinase activity.[147,148] Both studies proposed that active Ras would promote the formation of dimers. This hypothesis was later extended to heterodimerization between Raf-1 and B-Raf, which was found to be inducible by active Ras.[146] As mutation of S621 abrogated Raf heterodimerization, the authors speculated that 14-3-3 binding to pS621 was necessary for Raf heterodimerization. Although these initial studies showed that homodimerization and heterodimerization can activate Raf kinases, they failed to show that the interaction can take place between endogenous proteins when stimulated by physiological mitogens. This was achieved by Rushworth *et al*.,[82] who demonstrated that endogenous B-Raf and Raf-1 heterodimerize in multiple cell lines in response to mitogens. Biochemical fractionation of Raf heterodimers from homodimers and monomers showed that Raf-1–B-Raf heterodimers accounted for the majority of the mitogen-induced kinase activity. Remarkably, the heterodimers represented less than 1% of the total B-Raf pool but exhibited approximately 30-fold elevated kinase activity. Heterodimerization was enhanced by 14-3-3, but not a dimerization-negative 14-3-3 mutant, suggesting that the 14-3-3 dimer crosslinks Raf-1 and B-Raf by binding to the C-terminal sites on each kinase. This observation suggests a mechanism for how 14-3-3 can stabilize both inactive and active Raf-1 conformations. In the inactive conformation, 14-3-3 clasps the Raf-1 regulatory to the kinase domain via intramolecular binding to pS259 in the N-terminus and p621 in the C-terminus. Binding to activated Ras displaces 14-3-3 from pS259, leaving one 14-3-3 arm free to contact a 14-3-3 binding site in B-Raf to facilitate heterodimerization. In addition, Raf heterodimerization is also regulated by ERK-mediated feedback phosphorylation of B-Raf.[82,92] The feedback phosphorylation mainly serves to limit the lifetime of B-Raf–Raf-1 heterodimers, and mutation of the relevant sites enhances ERK signaling and the associated biological activities. Other regulators of Raf heterodimerization include KSR1[149] and MLK3,[150] which both enhance heterodimerization. MLK3 was originally described as an activator of JNK, but by activating B-Raf, it may serve as an integrating hub between the ERK and JNK pathways.[150]

Heterodimerization also may play a pathophysiological role in cancer. When B-Raf mutations were discovered in cancer,[20] a puzzling observation was that while the most frequent mutation, V600E, massively stimulated B-Raf kinase activity, several less frequent mutations activated B-Raf only mildly or not at all.[31] However, even the low-activity B-Raf mutants could hyperstimulate the ERK pathway. Intriguingly, this activation was dependent on the presence of Raf-1, suggesting that low-activity B-Raf mutants require Raf-1 to activate the ERK pathway. A subsequent study[81] confirmed the transactivation hypothesis by demonstrating that the low-activity B-Raf mutants found in cancer indeed could activate Raf-1 merely by forming dimers. While physiological heterodimerization is induced by Ras activation,[81,82] oncogenic B-Raf mutants constitutively dimerized with Raf-1.[81] The mechanism of activation by heterodimerization is incompletely understood. Mutational analysis showed that it does not depend on N-region phosphorylation but requires the activation loop phosphorylation sites.[81] This finding is difficult to reconcile with the observation that Ras-mediated activation of Raf-1 requires N-region phosphorylation.[70,75,106,151] The ability of Ras to induce Raf heterodimerization should overcome the requirement for N-region phosphorylation for Raf activation. Thus, more work will be needed to elucidate the mechanism of how heterodimerization activates Raf kinase activity. Regardless of the exact mechanism, dimerization clearly has biological effects. Increasing the lifetime of Raf heterodimers by mutating the T753 ERK feedback phosphorylation site in B-Raf augmented the ability of nerve growth factor (NGF) to induce neuronal differentiation of PC12 cells.[82] Furthermore, the mutation of ERK feedback phosphorylation sites and the concomitant increase in Raf heterodimer

levels enhanced the transforming potential of oncogenic B-Raf with intermediate or low kinase activity, which depend on Raf-1 for transformation, without affecting transformation by the high-activity B-Raf V600E mutant.[144]

An important role for Raf heterodimers was recently described in response to Raf kinase inhibitors. The original observation that Raf kinase inhibitors paradoxically could hyperactivate the Raf pathway[152] is now explainable by the ability of Raf kinase inhibitors to promote Raf heterodimerization and activation.[32,153,154] These findings are discussed below.

Signaling Downstream of Raf

Raf-Catalyzed MEK Phosphorylation

Despite much effort to identify Raf substrates, so far, the only *bona fide* physiological substrates of Raf are MEK1 and MEK2. Activated Raf kinases phosphorylate both MEK isoforms on 2 residues in the activation loop (S217 and S221). Phosphorylation on these 2 sites increases MEK activity, which in turn can bind, phosphorylate, and activate ERK (Fig. 1). Although all family members have the ability to bind and phosphorylate MEK *in vitro*, the activities towards MEK differ widely. B-Raf has the strongest activity towards MEK, followed by Raf-1 and A-Raf, whose MEK kinase activity is barely detectable.[106] B-Raf possesses higher basal activity partially because it is "primed" for activation due to the aforementioned constitutive phosphorylation of the S445 residue and the negatively charged amino acids at positions in the N-region that need to be phosphorylated in Raf-1 and A-Raf to achieve activation. Furthermore, of all Raf isoforms, B-Raf has the strongest binding affinity for MEK.[155] MEK is present in B-Raf protein-protein complexes even in starved cells, representing a preassembled complex ready to be activated.

The interaction of MEK with Raf-1 is also regulated by scaffolding proteins, such as KSR (see below), and by phosphorylation. The Rac effector kinase PAK1 can phosphorylate MEK1 on S298, which enhances the interaction with Raf-1[156] as well as ERK2.[157] Feedback phosphorylation of T292 by activated ERK prevents the phosphorylation of S298 and limits MEK activation by Raf-1.[158] This modulation is likely part of the biochemical basis for the cooperation between Ras and Rac proteins in cell transformation.[159,160] Structural studies revealed an interesting role for T292.[161] T292 only occurs in MEK1, but it can also downregulate the activity of MEK2 when T292-phosphorylated MEK1 heterodimerizes with MEK2. The implications of this differential regulation are not yet fully understood but indicate that MEK1 and MEK2 are not equivalent in terms of their regulation, and in a wider context, that dimerization of kinases also can exert negative control.

An unresolved question is why the activities of the comparatively poor MEK kinases Raf-1 and A-Raf are regulated in a much more complicated manner than the activity of the superior MEK kinase B-Raf. One possibility is that Raf-1 and A-Raf have other, yet unidentified substrates that are the real targets of their elaborate regulation. A more subtle possibility is that Raf-1 and A-Raf act as modulators that fine tune the ability of B-Raf to activate the ERK pathway. The discovery of B-Raf–Raf-1 heterodimers and their unfolding biological roles supports the latter view.

MEK-Independent Raf Signaling

A wealth of experimental data suggests that MEK is the only *bona fide* Raf substrate and that B-Raf is the main MEK kinase *in vivo*. This assessment is corroborated by phylogenetic comparisons. The single Raf homologs in invertebrates (lin-45 in *Caenorhabditis elegans* and D-Raf in *Drosophila*) are much closer to B-Raf in terms of sequence, suggesting that B-Raf is the archetypal MEK kinase, whereas A-Raf and Raf-1 may have been evolved towards MEK-independent functions.[162,163]

Alternative Raf substrates. Although MEK is the only commonly accepted Raf substrate, several other potential Raf-1 substrates were described. The adenylyl cyclases (AC) type 6, 5, and 2 were proposed to be phosphorylated and activated by Raf-1 independent of MEK.[164-166] As ACs generate cAMP, which activates PKA, their stimulation would initiate a negative feedback to Raf-1. Another proposed Raf-1 substrate is the retinoblastoma tumor suppressor protein (Rb)[167] (Fig. 4). The inactivation of Rb by cyclin-dependent kinases 4 and 6 marks the irreversible commitment of the cell to divide.[168] Raf-1 was reported to directly phosphorylate and inactivate Rb, leading to cell cycle progression.[167] Disruption of the Rb–Raf-1 complex by interfering peptides[169] or small molecules[170] suppressed the growth of experimental tumors and associated angiogenesis in nude mice. Two other potential Raf-1 substrates regulate myosin contractility. One is myosin phosphatase (MYPT), which binds to Raf-1 and is phosphorylated by Raf-1, leading to MYPT inhibition and enhanced cell motility.[171] Raf-1 can phosphorylate MYPT on the same site as the Rho effector kinase Rok-α and myotonic dystrophy protein kinase (MDPK).[171] Interestingly, both kinases are regulated by Raf-1; Rok-α via binding[19] and MDPK were reported to be phosphorylated and activated by Raf-1 directly.[172] Finally, Raf-1 was described to phosphorylate cardiac troponin T, which regulates the contractile function of cardiomyocytes.[173]

Lessons from raf gene knockout mice. However, while the functional role of these putative Raf-1 substrates is not yet clear, new Raf effector pathways were discovered in which Raf-1 kinase activity is dispensable and the regulation occurs through association (Fig. 4). These discoveries were facilitated by the availability of conventional and conditional *raf* knockout mice.[174] *a-raf* knockout mice are born alive but show neurological and intestinal defects of different severity depending on the genetic background.[175] In contrast,

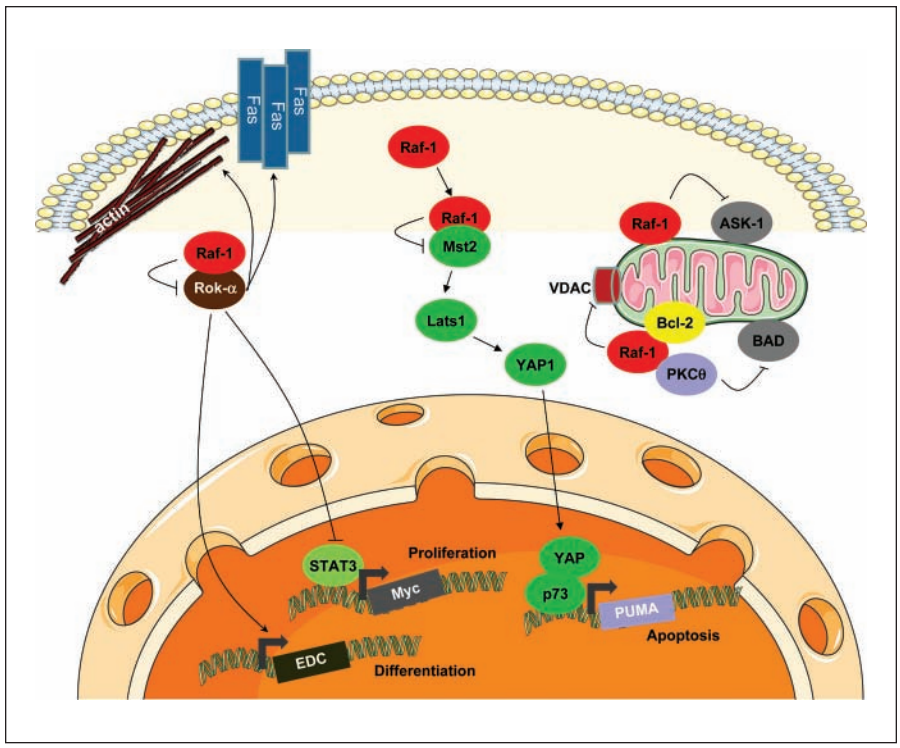

Figure 4. New Raf-1 signaling pathways that depend on protein interactions but not Raf-1 kinase activity or MEK. Raf-1 can suppress apoptosis in a MEK-independent fashion in several ways: 1) by binding to and inhibiting ASK1[16]; 2) by suppressing cytochrome C release through voltage-dependent anion channels (VDACs) at the mitochondria[195]; 3) by acting as a scaffold to recruit PKCθ to phosphorylate and inactivate BAD[194]; 4) by inhibiting the mammalian MST2 pathway[17]; and 5) by inhibiting Rok-α–induced Fas maintenance and clustering at the cell membrane.[18] In addition, the inhibition of Rok-α by Raf-1 is also required for motility by regulating the actin cytoskeleton[19] and for skin tumorigenesis by preventing keratinocyte differentiation and sustaining Myc expression.[214]

b-raf–deficient embryos die around midgestation because of vascular defects in the placenta. Epiblast-restricted ablation, which leaves *b-raf* intact in the placenta but knocks the gene out in the embryo, resulted in live born animals that within 3 weeks succumb to a violent neurodegenerative disease.[176] *raf-1*–deficient embryos show increased apoptosis of embryonic tissues or, more selectively, of the fetal liver depending on the genetic background.[177,178] These divergent phenotypes show that Raf-1, B-Raf, and A-Raf serve distinct essential functions in embryonic development. It subsequently became clear that a main function of Raf-1 was to restrict caspase activation in response to selected stimuli, notably Fas stimulation, pathogen-mediated macrophage apoptosis, and erythroid differentiation.[179,180] The ERK pathway can antagonize apoptosis in a number of ways, including the expression of caspase inhibitors and the neutralization of pro-apoptotic Bcl-2 family members.[181] A further prominent prosurvival molecule, the transcription factor NFκB, was proposed as a downstream target of Raf-1.[182-185] It is still unclear how Raf kinases activate NFκB, but it is likely through the induction of autocrine factors.[184,185] Importantly, neither MEK/ERK nor NFκB activation is altered in *raf-1*– or *a-raf*–deficient cells and embryos, indicating that the prosurvival roles of Raf-1 and A-Raf do not depend on these functions. What are then the essential downstream targets of Raf in apoptosis?

Raf-1 regulates apoptosis through multiple targets: Fas, ASK1, MST2, and Rok-α. A mitochondrial pool of Raf-1 was shown to protect cells from apoptosis.[186-188] Raf-1 could be targeted to the mitochondria via interaction with Bcl-2 when Bcl-2 was overexpressed.[189] In addition, selected growth factors were reported to promote Raf-1 translocation to the mitochondria via p21-activated kinase (PAK)–induced phosphorylation on S338.[190,191] However, it still is unclear how mitochondrially localized Raf-1 could prevent apoptosis. Raf-1 facilitates the phosphorylation and inactivation of the proapoptotic Bcl-2 family member BAD,[189] although this is likely an indirect effect mediated by ERK-activated RSK.[192,193] In addition, Raf-1 serves as a scaffold to recruit protein kinase C theta (PKCθ) to phosphorylate BAD.[194] Another described mechanism is the direct interaction between Raf-1 and mitochondrial voltage-dependent anion channels (VDACs), which may prevent the release of cytochrome C from the mitochondria.[195]

Gene ablation experiments in mice demonstrated that *Raf-1* is required for survival and protection against apoptosis.[177,178] Interestingly, reconstituting *Raf-1*$^{-/-}$ mice with a mutated Raf-1 (Raf-1 YY340/1FF), which has no detectable kinase activity towards MEK, fully rescued the apoptotic phenotype and produced viable mice.[178] Tracking the cause revealed several mechanisms, which may operate in a tissue-specific manner, but none of which requires Raf-1 kinase activity. One is the control of the Rho effector kinase Rok-α, which is hyperactivated and mislocalized to the membrane in *Raf-1* knockout cells.[18,19] Hyperactive Rok-α causes a defect in the internalization of the Fas death receptor, which maintains high levels of Fas in the plasma membrane, leading to increased Fas sensitivity.[18] The other targets of Raf-1 in apoptosis suppression are 2 proapoptotic kinases, ASK1[16] and MST2,[17] which are inhibited by Raf-1 through direct binding (Fig. 4). These inhibitions do not require Raf-1 kinase

activity but are solely mediated by binding. ASK1 is a protein kinase that works upstream of JNK and p38 to promote apoptosis induced by stress or by death receptors, such as the TNF-α receptor or Fas.[179,196] It was reported that in human endothelial cells, Raf-1 mediates the protective effect of basic fibroblast growth factor (bFGF) against doxorubicin-induced apoptosis by binding to and inhibiting ASK1 at the mitochondria. Mutation of S338/339 in the N-region abolished association and protection.[197] The mechanism of inhibition is not known, but the pathophysiological relevance of ASK1 inhibition by Raf-1 was demonstrated in a mouse model of heart disease.[198] Knocking out the *Raf-1* gene specifically in the heart muscle resulted in ventricular dilation and fibrosis caused by an increase in cardiomyocyte apoptosis. These pathological changes could be prevented by also knocking out ASK1.

The other Raf-1–inhibited proapoptotic kinase, MST2, was identified in a proteomics screen of Raf-1–associated proteins.[17] The mechanism of how Raf-1 regulates MST2 was elucidated. MST2 activation involves dimerization and autophosphorylation of the activation loop.[199] Raf-1 binds to the SARAH domain of MST2, thereby interfering with dimerization, and also recruits a phosphatase that dephosphorylates MST2.[17] Raf-1 kinase activity is not required, and kinase-dead Raf-1 mutants also can inhibit MST2 activation. Consequently, MST2 activity is constitutively elevated in Raf-1 knockout cells and hyperactivatable by Fas stimulation[17] or expression of RASSF1A.[200] RASSF1A can disrupt the Raf-1–MST2 complex and promote the assembly of a proapoptotic signaling complex consisting of RASSF1A, MST2, LATS1, and YAP.[200] In this context, MST2 phosphorylates LATS1, which phosphorylates YAP, thereby enabling YAP to interact with p73. The YAP-p73 complex binds to the promoter and activates the expression of the proapoptotic BH3 gene *PUMA*, culminating in the induction of apoptosis. This pathway was mapped using proteomics to track protein interactions that change in response to proapoptotic signals.[200] This study flagged important gaps in our understanding of MST2 signaling. First, it showed that the MST2 pathway in mammalian cells shares the core kinase module MST2-LATS but differs from the orthologous Hippo pathway described genetically in *Drosophila melanogaster* in regard to upstream regulators and downstream effectors. This finding was subsequently confirmed by other studies.[201,202] Second, it revealed the double nature of YAP as an oncogene as well as tumor suppressor. While in the liver, YAP is a potent oncogene,[203,204] YAP also can suppress oncogenesis by stimulating apoptosis in response to DNA damage[205-207] or expression of RASSF1A.[200] RASSF1A is a major tumor suppressor gene that is altered in the majority of human cancers usually by gene silencing due to promoter methylation and much less frequently by mutation.[208,209] Thus, being regulated by a major tumor suppressor pathway and a major mitogenic pathway, MST2 may have a critical role in coordinating apoptotic and transforming signals. Therefore, it is not surprising that MST2 is also targeted by the phosphatidylinositol 3-kinase (PI3K)/Akt survival pathway. Akt activation is required to curtail MST2 activation during growth factor stimulation. Akt phosphorylation of MST2 stimulates the dissociation of MST2 from RASSF1A and rebinding of MST2 to Raf-1.[210] The role of Raf-1 is reminiscent of the role of the Myc proto-oncogene, which can stimulate transformation and apoptosis.[211] Ras binding to Raf-1 enables Raf-1 to activate the MEK-ERK pathway and promote proliferation but at the same time dissociates the MST2–Raf-1 complex and promotes apoptosis.[212] Coupling cell proliferation to the risk of cell death seems paradoxical but makes perfect sense for a multicellular organism in which the unlicensed proliferation of cells can cause severe diseases including cancer. Interestingly, B-Raf fails to bind and regulate MST2.[17] Therefore, MST2 regulation by Raf is absent in *Drosophila melanogaster*, whose single Raf gene is most closely related to B-Raf. These observations suggest that MEK kinase activity was the primary function of Raf and that the ability to inhibit MST2 was acquired later in evolution.

Raf-1 regulates cell motility and differentiation through Rok-α. Another defect observed when *Raf-1* was knocked out specifically in keratinocytes was retarded wound healing and migration of keratinocytes.[19] Again, this novel function of Raf-1 can also be carried out by a kinase-dead mutant, and just like prosurvival, it involves the inhibition of another kinase. The target of Raf-1 in motility is Rok-α.[19] *Raf-1* knockout fibroblasts and keratinocytes have a contracted appearance, have a defective cytoskeleton characterized by tight cortical actin bundles, and fail to migrate. Chemical inhibition of Rok-α or expression of a dominant-negative Rok-α mutant rescues all these defects of the *Raf-1*–deficient cells, indicating that Rok-α is the only target of Raf-1 in motility.[19] Interestingly, investigating the mechanism of Rho-α inhibition by Raf-1 revealed a critical role for the Raf-1 CRD. Rok-α, like Raf-1, is regulated by autoinhibition, and its C-terminal regulatory region features a domain highly homologous to the CRD found in Raf-1. Indeed, dependent on an intact CRD, the Raf-1 regulatory domain could crossregulate Rok-α by binding to the Rok-α kinase domain and repressing its function.[213] The biological relevance of this interaction was borne out in a Ras-induced skin tumor model in mice.[214] In this model, the inhibition of Rok-α by Raf-1 was required for Ras transformation by maintaining the dedifferentiated state of the tumor cells.

A-Raf signaling independent of MEK. A-Raf is the family member with the poorest kinase activity towards MEK

and hence is likely to have functions outside of the classic ERK pathway. A-Raf strongly interacts with and inhibits MST2, again independently of kinase activity. Interestingly, this inhibition is contingent on the splice factor hnRNP H maintaining the expression of a full-length A-Raf protein.[215] hnRNP H is often overexpressed in tumors.[216] Downregulation of hnRNP H results in alternative splicing of the *a-raf* transcript that abolishes the expression of full-length A-Raf protein. Both A-Raf and MST2 are localized at the mitochondria in tumor cell lines as well as primary tumors.[215]

In a yeast 2-hybrid screen, pyruvate kinase M2 (PKM2) was identified as an A-Raf binding partner.[217] PKM2 is an embryonic splice form of PKM that is aberrantly re-expressed in cancer and responsible for the aerobic glycolysis, also known as the Warburg effect, in cancer cells.[218] A-Raf–mediated transformation increased the activity of PKM2 by promoting the transition of PKM2 from the low-activity dimeric to the highly active tetrameric form, and PKM2 enhanced A-Raf–induced cell transformation.[217,219] These findings potentially link A-Raf to the regulation of energy metabolism and cell transformation, a topic that is increasingly recognized as critical for tumorigenesis.[220]

Scaffolds and Modulators of Raf Signaling

For a long time, the Ras/ERK signaling pathway was depicted as a linear pipeline. Over the years, it became clear that signaling pathways form networks consisting of multiprotein nodes at various subcellular compartments.[24,221,222] A main question is how do these networks generate biological specificity? Much of this coordination depends on controlling protein-protein interactions by scaffold proteins that regulate the intensity, amplitude, and spatial specificity of the ERK pathway signal.[223,224] Scaffolds act as docking platforms and anchors of the signaling components, bringing together the different modules of the cascade. Thus, by facilitating interactions between their clients, they decrease reaction rates to the first order, and they also reduce the number of tiers of the cascade, causing the input/output responses to become more linear.[225] They insulate the clients from other pathways but also can connect pathways by binding components of different pathways. They can target their clients to different localizations, thereby increasing the variety of signals regulated by the cascade. There is now experimental evidence that scaffolds can link different localizations of Ras activation with the phosphorylation of specific ERK substrates.[226,227] Feedback phosphorylation of the EGF receptor (EGFR) by ERK involved the IQ motif containing GTPase-activating protein (IQGAP) scaffold, while the phosphorylation of cytosolic phospholipase A2 (cPLA2) utilized KSR1 or Sef-1 when ERK was activated by Ras localized at the plasma membrane or Golgi, respectively.[226] In addition, scaffolds seem to preferentially bind dimerized ERK and direct ERK to cytosolic substrates, whereas ERK dimerization is not required for the phosphorylation of nuclear substrates.[227] The requirement for ERK dimerization is likely related to the overlap between the binding sites for substrates and scaffolds, implying that a dimer is necessary to simultaneously engage the scaffold and substrate.[227]

Therefore, scaffolds can have a huge impact on the biochemical and biological behavior of the ERK pathway.[24,224] However, our knowledge of their role in the functional modulation of the pathway and their exact mechanism of action is still limited. One problem is that scaffolds are quite difficult to study experimentally. Their function is highly dependent on concentrations and the stoichiometric ratios with respect to their client proteins, and both downregulation and overexpression have similar effects, as both conditions reduce the number of functional complexes. Scaffold proteins of the ERK pathway were extensively rev iewed.[24,223,224,228-234] We, therefore, only describe selected examples that allow us to outline salient functions of scaffolding proteins in the regulation of the ERK pathway (Fig. 5). For convenience, we have classified them by their major known functions. However, occasionally, these functions overlap and will expand as more details become known.

Scaffolds as Regulators of ERK Pathway Activity

The best-characterized scaffold of the ERK pathway is **kinase suppressor of Ras 1 (KSR1)**. Initially identified as a suppressor of an activated Ras phenotype in *Drosophila melanogaster*[235] and *Caenorhabditis elegans*,[236,237] KSR1 has a kinase domain with high homology with Raf-1 but mutations in residues critical for catalytic activity. Whether KSR1 has remaining kinase activity or whether it is a pseudokinase is still discussed in the literature.[234,238,239] However, it is now accepted that the main function of KSR1 is as a scaffold of the ERK pathway, which regulates the intensity and duration of the ERK signal independent of catalytic function. KSR1 can interact with all kinases of the ERK pathway. MEK is constitutively bound, while Raf (Raf-1 or B-Raf) and ERK are recruited to KSR1 upon mitogen stimulation.[80,240,241] However, KSR1 only binds less than 5% of endogenous Raf-1,[242] indicating that KSR1 affects only a subset of Raf functions, and Raf members might be present in other protein complexes. KSR1 can activate *Drosophila melanogaster* Raf (which is closely related to mammalian B-Raf) allosterically by dimerization. In addition, KSR1 facilitates N-region phosphorylation of Raf-1 and B-Raf by recruiting CK2.[77] Thus, KSR does not only regulate substrate availability but also catalytic activity, suggesting complex kinetic effects. In the context of cancer biology, KSR1 regulates Ras-mediated signaling, in particular differentiation, proliferation, and cellular transformation.[243-246] Gene deletion of KSR1 in the mouse had little effect on viability but decreased the oncogenic effects of the polyoma virus middle T antigen[247] and blunted oncogenic Ras-mediated

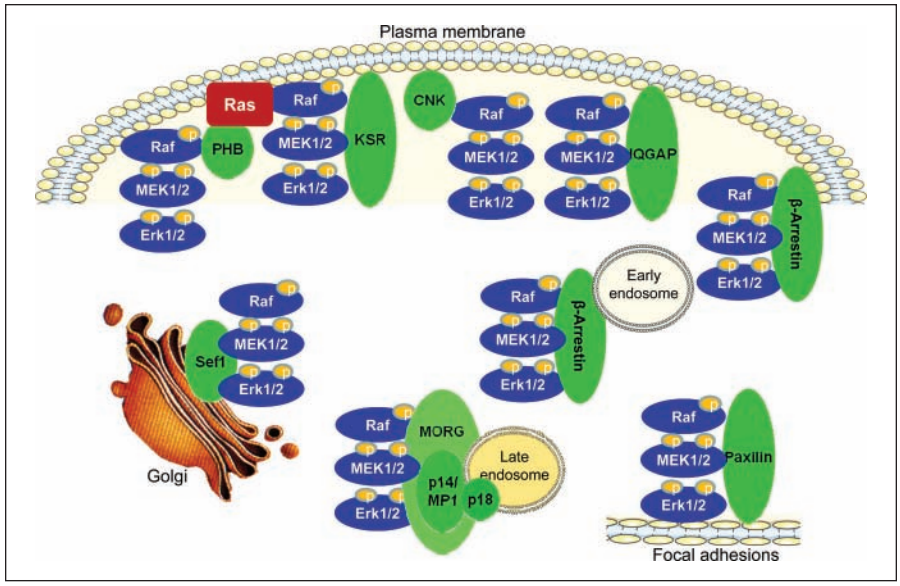

Figure 5. Scaffolding proteins in Raf-MEK-ERK signaling. Scaffolding proteins form Raf-MEK-ERK signaling platforms at different subcellular localizations. See text for details.

tumorigenesis.[248,249] KSR1 depletion also accelerated the immortalization of cells and led to resistance to cisplatin-meditated apoptosis, while overexpression of KSR1 sensitized tumor cells to this anticancer agent and other drugs.[250,251] Taken together, the functions of KSR1 in the context of Ras/ERK signaling vary dramatically and depend, like other scaffold proteins, on the level of expression. At low and physiological levels, KSR1 seems to work as a positive regulator of signaling. By contrast, overexpression of KSR1 has an inhibitory function on the activation of the ERK cascade.[233,234] Recently, KSR2, a homolog of KSR1, was shown to participate in the calcium-mediated activation of ERK.[252] This regulation is exerted by the calcium-dependent phosphatase calcineurin, which binds to KSR2 and dephosphorylates it, resulting in increased membrane translocation and ERK signaling. Another proteomic study showed that KSR2 preferentially interacts with A-Raf in response to TNF-α.[253] The biological relevance of this interaction remains to be elucidated, but as KSR2 knockout mice have a striking metabolic dysregulation that includes obesity,[254,255] it is tempting to speculate that KSR2 may be involved in mediating A-Raf effects on metabolism.

Another group of Raf scaffolds is the **connector enhancer of KSR (CNK)** family of proteins. First identified as a modifier of KSR signaling in *Drosophila melanogaster* dCNK,[256] mammals possess 3 isoforms that lack kinase activity but feature different protein-protein interaction domains that can bind a variety of client proteins including Raf-1 and B-Raf.[224,234] Thus, CNK proteins seem to be superscaffolds that may integrate different signaling pathways. It is beyond the scope of this review to discuss CNK function in detail, and hence, we only will focus on the role of CNKs in Raf regulation. In *Drosophila melanogaster*, a multiprotein complex formed between dCNK, Raf, KSR, and a small adaptor protein HYP mediates Ras-induced activation of Raf.[257,258] HYP has no mammalian homolog, and mammalian CNKs lack the Raf regulatory domain found in dCNK, suggesting a different mode of regulation. Mammalian CNK2 participates in the NGF-induced sustained ERK activation that is required for the neuronal differentiation of PC12 cells.[259] The mechanism was not defined but, by analogy to CNK1, may involve facilitation of Raf activation. CNK1 can augment Raf-1 activation by increasing tyrosine phosphorylation of the N-region through recruiting c-Src.[260] Interestingly, CNK1 also can bind RASSF1A and enhance apoptosis in a MST1/2-dependent manner.[261] Thus, it is tempting to speculate that CNK1 may play a role in balancing apoptosis and proliferation by coordinating MST2 binding to RASSF1A and Raf-1, respectively.

In addition to KSR1, other ERK pathway scaffolds were implicated in tumor progression. Among them are the **IQ motif containing GTPase-activating proteins (IQGAPs)**, a family of multidomain proteins.[262,263] IQGAP1 directly interacts and modulates the functions of B-Raf, MEK, and ERK.[264,265] Furthermore, IQGAP1 is required for the activation of B-Raf by EGF.[266] As a result, IQGAP1 increases proliferation and reduces cellular differentiation. Thus, it comes as no surprise that IQGAP1 is involved in carcinogenesis.[230,263] Augmented expression of IQGAPs was reported for several malignancies including cancers of the stomach, colon, lung, and prostate.[263] Overexpression of IQGAP1 in human breast epithelial cells increased the formation and invasion of tumors, whereas reducing IQGAP1 expression had the opposite effect.[267] Therefore, IGGAP1 is considered as a putative oncogene.[268] Furthermore, IQGAPs are linked with metastasis, as IQGAP1 promotes cell migration and invasion via direct interactions with Cdc42, Rac1, actin, and calmodulin.[263]

Prohibitin (PHB) facilitates the displacement of 14-3-3 from Raf-1 by activated Ras, thereby promoting plasma membrane localization and phosphorylation of Raf-1 at the activating S338.[269] Interestingly, PHB binds only to Raf-1, but not to Ras, and may function as a chaperone of Raf to enable interaction with Ras. In the context of cancer, PHB function was connected to immortalization, aging, and cell cycle regulation.

Furthermore, PHB overexpression was reported in breast cancer, human endometrial adenocarcinoma,[270] gastric cancer,[271] and bladder cancer,[272] although it is not known if this is related to aberrant Raf function.

Scaffolds as Spatial Regulators of the ERK Pathway

Scaffold proteins are also crucial for the localization of the members of the ERK pathway to different subcellular signaling platforms. One such scaffold is **similar expression to FGF (Sef-1)**, also known as interleukin-17 receptor (IL-17RD), which is situated at the Golgi apparatus.[273] This transmembrane protein binds to activated MEK and facilitates activation of ERK but prevents ERK translocation to the nucleus. Therefore, ERK can only activate cytosolic targets. Interestingly, loss of Sef-1 expression is associated with high-grade metastatic prostate cancer.[274] In clathrin-coated pits, the **β-arrestins** were proposed to augment ERK activation by scaffolding Raf-1, MEK, and ERK.[275,276] The β-arrestins seem to act in a similar fashion as Sef-1, preventing ERK nuclear translocation and therefore restricting Ras signaling to the cytoplasmic effectors of the pathway.

The small scaffold **MEK partner-1 (MP1)** is an obligatory heterodimer with p14, and this complex interacts with MEK and ERK, targeting them to late endosomes.[277,278] Recent results suggest that an additional adaptor, p18, is involved in specifying this subcellular localization.[279] *In vitro* results indicated that MP1 also may facilitate MEK activation by B-Raf, although the mechanism is unknown.[280] While decreased MP1 levels reduce ERK activation, overexpression of MP1 increases the binding of ERK to MEK and thus enhances the efficiency of ERK signaling.[278,280] The speculation that the specific localization directed by MP1 generates signaling specificity was confirmed by the finding that MP1 specifically regulates PAK1-mediated ERK activation during cell adhesion and spreading but is not required for ERK activation by PDGF.[281] Enhanced MP1 expression in several melanoma cell lines could be linked with a genetic translocation (4q23),[282] suggesting a mechanism for the enhanced MAPK signaling in melanomas. In addition, MP1 may target MEK-ERK to high molecular weight protein complexes.[283] Such complexes may be organized by **MAPK organizer 1 (MORG1)**, a member of the WD-40 protein family, which was identified as an interaction partner of MP1 as well as Raf-1, B-Raf, MEK, and ERK.[284] There is evidence that MP1 and MORG1 are part of a larger network built from nested scaffolds,[283] as MEK binding to MORG1 is stabilized by MP1, Raf-1, and ERK. MORG1 acts like a classic scaffold with enhanced activation of ERK at low concentrations and being inhibitory at higher concentrations.[284] Furthermore, MORG1 promotes ERK activity in response to serum or other signals. Interestingly, MORG1 was also shown to act as a scaffold with hypoxia-inducible factor prolyl hydroxylase 3 (PHD3) and downregulation of MORG1-augmented HIF-1 activity, suggesting MORG1 as a connection to other signaling networks.[285]

The multidomain protein **paxillin** is a component of focal adhesions, providing a structural and signaling link between the actin cytoskeleton and the extracellular matrix (ECM).[286] Paxillin constitutively interacts with MEK but in response to growth factors also binds to activated Raf and ERK, directing activated ERK to sites at the focal adhesions.[287] The most significant impact of paxillin is on developmental processes and on tissue morphogenesis,[287-289] but it also plays a role in tumor cell invasion.[290] Elevated levels of paxillin, together with enhanced Src activity, contribute to the high metastatic potential of human osteosarcomas, and in gastric cancer, high levels of paxillin correlated with advanced tumor stage and invasiveness.[291]

Modulators of Protein Interactions in the ERK Pathway

Raf kinase inhibitor protein (RKIP) is receiving sharply increasing attention as a modulator of the ERK pathway and several other signaling pathways including G protein signaling and NFκB signaling.[224,292-294] Initially identified as phosphatidylethanolamine-binding protein-1 (PEBP-1), RKIP was later identified as a negative modulator of Raf-1.[91] RKIP binds Raf-1, MEK, and ERK. While RKIP can bind MEK and ERK simultaneously, binding to Raf-1 and MEK is mutually exclusive, disrupting the Raf-1–MEK complex and activation of MEK by Raf-1.[90] RKIP also interferes with Raf-1 activation by preventing the interaction with PAK1 and Src kinases and the phosphorylation of the N-region.[295] In this study, B-Raf activation was not affected. However, another study found that RKIP inhibited B-Raf activation in cells as well as its ability to phosphorylate MEK *in vitro*.[296] Interestingly, in an RKIP-related protein, **hPEBP4**, the Raf- and MEK-binding sites, which overlap in RKIP, are separated by an insertion converting hPEBP4 into a scaffold for the Raf-1–MEK complex.[105] Consequently, hPEBP4 enhances the activity of the ERK pathway in growing human myoblasts. Upon induction of differentiation, hPEBP4 expression rises to levels that exceed the optimal stoichiometric relationship to its client proteins and contribute to the inhibition of the ERK pathway observed during myoblast differentiation.[105] This regulation highlights not only that scaffolds can assume both stimulating and inhibitory roles under physiological conditions but also that a primary function of such proteins is the fine tuning of the activation kinetics of signaling pathways. RKIP has a main function in causing switchlike activation behavior of ERK and in supporting oscillations of the pathway caused by the negative feedback from ERK to Ras activation.[297] Physiologically, RKIP plays a major role as a suppressor of cancer invasiveness and metastasis in various cancers[294] including common cancers of the prostate,[298] breast,[299] colon,[300] and liver.[301]

Other negative modulators of Raf-mediated signaling are members of the **Sprouty (Spry)** family, comprising

various Spry and Spred (Spry-related proteins with an EVH1 domain) isoforms.[302-306] These proteins are negative feedback regulators of the ERK signaling pathway, and their expression is regulated by the cascade. Depending on the context, Spry proteins inhibit ERK signaling by binding and sequestering the Grb2-SOS complex, thus preventing Ras activation.[307] Additionally, both Sprouty and SPRED can physically interact with Raf-1 and B-Raf, interfering with the phosphorylation of Raf on activating sites.[308-310] Spry genes and proteins were shown to be deregulated in different tumor types. Spry1 and Spry2 are downregulated in breast, prostate, and liver cancer.[223,310,311] This downregulation seems to be due to hypermethylation of the promoter region, indicating that Sprys are putative tumor suppressors.

Genetic Alterations in Raf Family Genes

Raf Mutations in Cancer

For almost 2 decades, research focused on Raf-1 as the critical Ras effector of the Raf family. However, this changed when Davies *et al*. described mutations of B-Raf in 66% of melanomas and at a lower frequency in a wide range of human solid cancers.[20] Further research revealed that approximately 2% of human malignancies carry a mutation in B-Raf,[312] with highest frequencies observed in melanoma and carcinomas of the colon, thyroid gland, ovary, and biliary tract.[21,313,314] Currently, more than 100 different B-Raf mutations were described, with V600E (formerly labeled as V599E) being by far the predominant lesion (Catalogue of Somatic Mutations in Cancer: www.sanger.ac.uk/genetics/CGP/cosmic). Most of the B-Raf mutants associated with cancer are located in exons 11 or 15 in the kinase domain. The biggest group of mutations (including V600E) affects residues that normally stabilize the kinase in the inactive form. Mutations of these amino acids disrupt this conformation, usually resulting in a significantly increased B-Raf kinase activity that leads to the constitutive activation of the ERK pathway.[31] However, even impaired kinase activity mutants can constitutively activate the ERK pathway because of their ability to heterodimerize with and activate Raf-1.[31,81] Interestingly, B-Raf mutations normally do not coexist with oncogenic mutations in Ras in human tumors, arguing that they are equivalent in their transforming effects.[315] This conclusion indeed highlights B-Raf as a critical effector of Ras in cell transformation and cancer. In further support of this interpretation, mutant B-Raf has also been shown to be a critical step in tumorigenesis in mouse models of melanoma.[316,317] Melanocyte-specific, conditional expression of the V600E mutation inserted into the endogenous *BRAF* gene locus resulted in the development of both benign nevi and malignant melanoma. This is in line with observations from human tumors, where B-Raf V600E is detected in approximately 44% of melanoma cases,[316] but with even higher frequency in benign nevi, which do not progress into a malignant melanoma.[318] This dormancy is probably due to the potent induction of senescence by B-Raf V600E, which suppresses tumorigenesis.[316,319] These data suggest that additional secondary alterations cooperating with mutant B-Raf may be required. Further support of this hypothesis comes from observations demonstrating that B-Raf V600E induces senescence and needs secondary events like p16INK4A loss to overcome it.[319] Interestingly, melanomas in the above-mentioned mouse model did not show any alterations in p16INK4A, suggesting that the pathogenetic mechanisms differ between the mouse and human or else that there are other, hitherto unknown possibilities for secondary genetic events. However, there are also B-Raf mutations associated with human cancer, which display impaired kinase activity, with D594V being the most frequent one. These mutations require Raf-1 to activate the ERK pathway,[31] relying on the ability of catalytically compromised B-Raf to activate Raf-1 by heterodimerization.[81,82] As Raf heterodimerization is augmented by activated Ras, these low-activity B-Raf mutants (in contrast to the high-activity mutants) can be found coexpressed with mutant Ras.[154]

Although quite rare, cancer-associated mutations also were reported in Raf-1. They were first described in a mouse model of chemically induced lung cancer,[320] in human cancer cell lines,[321] and finally in patients with therapy-related acute myeloid leukemia (t-AML).[322] Interestingly, the latter mutations were detected in the germline of affected patients but still exhibited weakly transforming and antiapoptotic properties. Therefore, they might constitute a hereditary predisposition to solid neoplasms and t-AML. This hypothesis was supported by the observation that constitutive activation of the ERK pathway in affected patients was only observed in malignant but not in the surrounding normal tissues. Indeed, further studies identified a leukemia-specific, somatic loss of RKIP as a genetic second hit that further promoted malignant transformation in these patients.[323]

Besides mutations, other alterations of Raf genes were described in human malignancies as well. Rearrangements and fusions of B-Raf and Raf-1 to a variety of other genes have been described in thyroid cancer, pilocytic astrocytoma, prostate cancer, gastric cancer, and melanoma.[324-328] They seem to be particularly frequent in sporadic pilocytic astrocytoma, with more than 60% of cases demonstrating B-Raf rearrangements.[324] Usually, the resulting fusion products lose the regulatory N-terminal region but retain an intact Raf kinase domain. They can activate the ERK pathway, transform transfected cell lines, and induce tumors in nude mice, underlining their functional role in the pathogenesis of human malignancies. Mutated B-Raf further was found amplified in human melanoma with a gain of chromosome 7q. This amplification is a

frequent event in melanoma, suggesting that B-Raf mutations are one of the factors driving its selection.[329] Elevated levels of A-Raf mRNA and protein were observed in a number of malignancies including head and neck squamous cell carcinomas and colon carcinomas.[215] This study also showed that the splice factor heterogeneous nuclear ribonucleoprotein H (hnRNP H) is required for the correct splicing and expression of full-length A-Raf. Elevated expression of A-Raf was found in testicular germ cell tumor–derived cell lines caused by the duplication of the X chromosome.[330]

Raf Mutations in Developmental Syndromes

Raf mutations are not only critical steps for tumorigenesis but also for the pathogenesis of rare developmental disorders, such as neurofibromatosis type 1 and Costello, Noonan, LEOPARD, and cardiofaciocutaneous (CFC) syndromes, which are reviewed in detail elsewhere.[331-333] Affected individuals present with overlapping yet distinct phenotypes that include a variable degree of mental retardation, cardiac defects, facial dysmorphisms, short stature, macrocephaly, and skin abnormalities. Germline mutations in Raf were first linked to these disorders when 2 groups simultaneously reported mutations in B-Raf causing CFC.[334,335] The B-Raf mutations in CFC comprised mainly hitherto unknown mutations, which were more widely distributed across B-Raf as compared to their counterparts detected in human cancer. However, a few mutations are shared between cancer and CFC. Some of the CFC germline mutations resulted in increased B-Raf kinase activity and constitutive activation of the ERK pathway.[336] Compared to the V600E mutation, the kinase activity and transforming capacity of CFC B-Raf mutants seem to be lower.[333,337]

Germline mutations in Raf-1 were recently described in both Noonan and LEOPARD syndromes.[338,339] As with B-Raf, one of the mutations (S427G) was found in both Noonan syndrome and human malignancies, and again, some of the germline variants increase Raf-1 kinase activity and transforming ability.[322,340] One might expect that carrying an oncogene in the germline increases the risk for the development of malignancies. Indeed, patients with neurofibromatosis type 1, Costello syndrome, and Noonan syndrome are at increased risk for developing a wide range of solid tumors and hematological malignancies.[331-333] Whether CFC and LEOPARD syndromes result in a predisposition to tumor development is an open question. The numbers of patients affected by these disorders are too low for a thorough statistical analysis, and descriptions of patients developing a malignant disorder are limited to case reports.[341-345] However, close monitoring of patients with all germline Ras/MAPK disorders, including LEOPARD and CFC syndromes, for the occurrence of neoplasias is often suggested.

Targeting Raf and MEK for Cancer Treatment

The efforts to develop drugs targeting the Raf family and their downstream effectors were increased after strategies implemented to inhibit Ras signaling failed in the preclinical and clinical studies.[346] Different approaches included the inhibition of Raf kinase activity by small molecule inhibitors, decreasing Raf protein levels using antisense oligonucleotides, and targeting Raf protein-protein interactions, especially the Raf-Ras interaction. The first drugs were developed against Raf-1, but the discovery of B-Raf–activating mutation in tumors[20] shifted the efforts toward the inhibition of this protein and of MEK1/2.

Raf Inhibitors

Sorafenib (BAY 43-9006) was the first Raf inhibitor to progress into clinical trials. Preclinical studies showed that sorafenib inhibited Raf-1 and B-Raf in tumor cell lines and xenograft models for Ras-dependent tumors.[347,348] Today, sorafenib is approved for the treatment of advanced renal cell carcinoma (RCC) and unresectable hepatocellular carcinoma (HCC). However, sorafenib monotherapy failed to be clinically effective against other tumors such as melanoma, although it increased progression-free survival in combination with other treatments.[349,350] Sorafenib was developed as a specific Raf-1 kinase inhibitor, and while it poorly inhibits mutant B-Raf, it is highly effective against several other kinases such as VEGF and PDGF receptors.[351] In fact, sorafenib is now regarded as a multikinase inhibitor, and the success in HCC and RCC is probably primarily because of the inhibition of VEGF and PDGF receptors rather than Raf kinases. The results of the sorafenib clinical trials raised questions about the suitability of Raf proteins as therapeutic targets and the low predictive properties of the preclinical models. One possible explanation for the unexpected therapeutic target spectrum of sorafenib is that it is a poor B-Raf inhibitor. This conclusion led to the development of a new generation of inhibitors active against B-Raf. **RAF265** is active against all Raf isoforms, mutant B-Raf, and VEGF-2. RAF265 inhibits cell proliferation in mutant B-Raf and N-Ras melanoma cells but has no effect in cell lines that express the normal genes.[352] This finding led to the initiation of a series of clinical trials for the treatment of advanced melanoma in which patients were evaluated for B-Raf and N-Ras mutation before treatment. The results from these clinical trials are eagerly awaited and are expected to be published shortly. **XL281** is another pan-Raf inhibitor that is active against mutant B-Raf and currently is in phase I clinical trials. Finally, **PLX4032** is a potent B-Raf V600E selective kinase inhibitor that suppresses the activation of the ERK pathway and cell proliferation in melanoma xenografts.[353,354] PLX4032 does not inhibit the ERK pathway in cells that do not express mutant B-Raf. A recent clinical phase I study reported a spectacular response rate in 81% of melanoma patients with mutant B-Raf.[355] These results demonstrate that mutant B-Raf is

an excellent therapeutic target in melanoma. However, this success did not come without cost, as 31% of patients developed skin tumors, keratoacanthomas, and squamous cell carcinomas. This side effect may be due to the enhancement of aberrant ERK pathway activation by drug-induced B-Raf–Raf-1 dimerization as discussed below. Although the skin tumors can be easily recognized and surgically removed, the appearance of malignancies in other less accessible organs remains a concern, and further studies are advocated before PLX4032 is approved for the treatment of metastatic melanomas.

The generation of drugs that target Raf interactions with other proteins is not as advanced but conceptually promising. **MCP-110** is a small molecule that inhibits the Raf-Ras interaction. This compound decreased anchorage-independent growth in cell lines expressing oncogenic Ras but did not affect cell lines with a constitutively active Raf-1, indicating that MCP-110 is working specifically at the level of the Ras-Raf interaction.[356] However, this agent did not progress into preclinical development. Another Raf interaction that was targeted is the binding to the Rb tumor suppressor protein. As mentioned above, Raf-1 was reported to bind to and phosphorylate Rb, resulting in Rb inhibition and S phase progression.[357] Small synthetic peptides that interrupt the Raf-1–Rb interaction in cell lines suppressed the growth of A549 xenograft tumors.[169] The use of peptides as drugs is limited by their short half-life and problems in their delivery, but similar results were obtained using the small-molecule drug **RRD-251**. This drug inhibited cell proliferation *in vitro* and suppressed the growth of xenograft tumors as well as tumor angiogenesis in a manner dependent on the expression of intact Rb.[170] Although no clinical data are available, these results indicate that targeting the Raf-1–Rb interaction may be a successful antitumoral strategy. It also suggests that targeting other Raf interactions may be a good strategy. Of special interest would be the disruption of Raf-1 binding to Rok-α, which may promote the differentiation of epidermal skin tumor cells,[214] and the dissociation of Raf-1 from MST1/2[17] and ASK1,[16] which should activate the proapoptotic potential of these kinases. More interestingly, the disruption of the Raf-1/A-Raf–MST1/2 complex should compensate for the frequent loss of the RASSF1A tumor suppressor, which normally promotes the disruption of the MST–Raf-1 complex and activation of MST1/2.[200] Such drugs would restore a natural tumor suppressor function and hence may be expected to be specific, efficacious, and without severe side effects.

A greater advance was made in targeting RAF expression using antisense oligonucleotides. The use of oligonucleotides for therapy is restricted by their susceptibility to degradation by nucleases, but the substitution of oxygen for sulfur in the phosphodiester linkages confers stability to these molecules.[358] Using these chemical modifications, different compounds were generated, such as **ISIS 5132** and **ISIS 2503**. **ISIS 5132** is a 20-base phosphorothiate antisense oligonucleotide against Raf-1 that inhibited tumor progression in clinical trials. However, subsequent phase II clinical trials demonstrated that this compound was of no benefit as a single agent, and hence, it was not further developed.[356] Another Raf-1–directed antisense agent that entered clinical trials is **LErafAON**, a liposome-entrapped derivative of a 15-mer antisense oligonucleotide.[356] Packaging the antisense oligonucleotide within liposomes was expected to protect the oligonucleotide against nucleases and increase cell delivery. Unfortunately, several phase I clinical trials demonstrated a lack of objective responses but adverse side effects due to the liposomal formulation.[359,360] The failure of the antisense drugs suspended the development of this approach for Raf inhibition. However, improvements in delivery methods,[361] and the observation that small interfering RNA (siRNA) against mutant B-Raf[362,363] and Raf-1[364] can inhibit cell proliferation in melanoma and breast cancer xenograft models, respectively, may rekindle the interest in this strategy.

Paradoxical Effects of Raf Inhibitors

A decade ago, a report was published showing that the Raf inhibitor ZM 336372 produced a massive paradoxical activation of Raf kinases when cells were treated with the inhibitor, and then, Raf kinases were isolated and their activity measured *in vitro*.[152] Now, 3 publications finally shed light on this apparent contradiction.[32,153,154] The key is Raf-1 homodimerization or heterodimerization with B-Raf, which is driven by mutant Ras and facilitated by the Raf inhibitor drugs. The activation conferred by dimerization is not compromised if the kinase activity of one of the Raf dimerization partners is destroyed and the kinase activity of Raf-1–B-Raf heterodimers is higher than that of Raf-1 homodimers.[82] Dimerization, induced by Ras or by Raf inhibitors, of mutant B-Raf V600E and Raf-1 actually dampens the overall kinase activity.[365] These constellations exacerbate the activation effect when B-Raf–specific inhibitors are used and Raf dimerization is ushered by Ras mutations. Thus, Raf inhibitors are rather effective when B-Raf is mutated but ineffective when Ras is mutated.[32,153,154] Beyond this shared theme, the 3 studies differ in mechanistic details.

In cell lines expressing high-activity B-Raf mutants, Raf inhibitors function as expected and efficiently reduced signaling through the ERK pathway.[154] Consequently, cell proliferation was inhibited *in vitro* as well as in xenografts when cells were treated with B-Raf inhibitors. Surprisingly, in cell lines lacking an activating B-Raf mutation and expressing mutant Ras, the effects were opposite.[32,154] Specific B-Raf inhibitors, such as PLX4720, enhanced ERK phosphorylation, and the cells demonstrated a high drug tolerance in xenograft models and proliferation assays. Moreover, the inhibitors initiated Raf heterodimerization, which enhanced the kinase activity and

downstream signaling. Pan-Raf and less specific inhibitors, on the other hand, enhanced ERK signaling at a lower concentration, while higher doses reverted this effect and caused inhibition.[153] These data suggest that both Raf-1 and B-Raf kinase activities present in a dimer need to be inhibited in order to shut down the signaling efficiently. Consistently, the inhibitor-induced ERK activity is Raf-1 dependent when B-Raf–specific inhibitors are used.[32,153] Interestingly, B-Raf inhibitors are able to activate Raf-1 independently of B-Raf.[153] PLX4720 robustly induces ERK phosphorylation in B-Raf$^{-/-}$ fibroblasts, which led to the conclusion that association with the inhibitor and drug-induced Raf-1 homodimerization is sufficient to enhance Raf kinase activity and ERK signaling. Heidorn *et al*.[154] could also demonstrate in a mouse model that mutant K-Ras and kinase-dead B-Raf cooperated in the induction of melanomas. This is a worrying discovery, and it would be interesting to know if B-Raf inhibitors would have the same effect as kinase-dead B-Raf in promoting melanoma in a mutant K-Ras model.

In light of these data, the development of keratoacanthomas and squamous cell carcinoma in 31% of melanoma patients treated with PLX4720[355] may be ascribed to the paradoxical activation of Raf signaling by drug-induced heterodimerization. These results have direct implications for clinical practice. Firstly, the patient population, which should receive B-Raf–specific inhibitors, has to be carefully selected, as only tumors expressing activating B-Raf mutations will respond to the treatment, while mutant Ras expression may even worsen the condition. Furthermore, a combination therapy including pan-Raf and MEK inhibitors should also be considered in order to enhance the efficacy (see below) and limit adverse side effects and the fast onset of drug resistance.

MEK Inhibitors

The first highly selective and effective inhibitors against the ERK pathway were directed against MEK1/2. Work from different groups in preclinical models indicated that MEK inhibitors are highly effective if the pathway is activated by B-Raf mutations but rather ineffective against cells harboring mutant Ras.[366,367] **CI-1040**, the first MEK inhibitor to proceed to clinical phase I trials, had limited side effects and reduced ERK phosphorylation in more than 65% of the tumor biopsies.[368] However, a subsequent phase II trial did not show clinical responses in a variety of tumor entities,[369] and consequently, the development was stopped. Nevertheless, these studies suggested that targeting MEK was relatively safe, and new generations of MEK inhibitors were developed.[370] **PD0325901**, one of these new-generation MEK inhibitors, is 50 times more potent than CI-1040 and has better pharmacological properties. However, the results from 2 clinical trials were disappointing, showing a high level of toxicity, and PD0325901 was dropped from further development.[348] Currently, 7 MEK inhibitors are in different phases of clinical trials, and many more are in preclinical development.[370] The results published so far show different antitumoral efficacies and also different levels of toxicity.

Thus, the development of drugs against the Raf family and MEK has produced both successes and disappointments, which have helped to develop new strategies that may lead to better compounds or better use of compounds. Most of the Raf and MEK inhibitors show limited effect as single agents, but they may still be effective in combination with classic chemotherapeutic agents or drugs that specifically target other signaling pathways, such as PI3K or growth factor receptor inhibitors. Deriving effective drug combinations will be helped by the rational prediction of drug responses using mathematical and computational modeling (see below) and by the identification of better biomarkers that allow discriminating, which patients would benefit from the treatment with Raf or MEK inhibitors, leading to the creation of personalized protocols for individual patients.

A Systems Biology View of the Ras-Raf Signaling Network

Despite the 25 years of research on Ras and Ras effectors, we are still bewildered by the diverse functionalities of this pathway, which can specify a multitude of often contradictory biological outcomes. While we have identified a large number of components and thoroughly characterized the individual functions of many of them, we still lack an understanding of how the Ras-Raf network processes signals to generate specific biological responses. Emerging evidence shows that much of this specificity is encoded by the pathway structure and dynamic changes in the connections between the proteins.[24,126,297,371,372] These so-called emergent properties are difficult to understand by experimentation alone. Therefore, mathematical and computational modeling approaches were developed that allow us to analyze and predict network responses including the behavior of the Ras-Raf pathway.[24,373]

The Cascade Structure of Ras-Raf Signaling Networks: Amplification of Signals and Sensitivity

The "backbone" of the Raf pathway consists of a 3-tiered kinase cascade. This design allows for a larger repertoire of regulation by feedback, crosstalk, and scaffolding to increase the number of signaling functions a single pathway might perform. It also allows for signal amplification, turning a low-abundance, noisy signal into a higher abundance, clearer signal at each tier of the cascade. Measured in terms of active molecules, the amplification factor from active Ras to active ERK can be 6- to 30-fold.[374-376] Not only does this design amplify signals, but it also amplifies the sensitivity of the cascade output (ppERK) to the cascade input (RasGTP).[377,378] This property was also shown experimentally in *Xenopus* oocytes, where the effective cooperativity (Hill coefficient) increases

with each tier of the cascade, so that a defined increase in the stimulation of the first component causes successively larger increases downstream.[379] The activation mechanism by multisite phosphorylation adds further to this sensitivity amplification, if the phosphorylations occur in a distributive manner, where each phosphorylation requires a separate binding event between kinase and substrate.[380,381] *In vitro* ERK phosphorylation by MEK and dephosphorylation by MAPK phosphatase 3 (MPK3) follow such a distributive mechanism,[382,383] which based on theoretical considerations can give rise to switchlike behavior, bistability, and oscillations.[380,384] While bistability of ERK activity was experimentally observed in several settings,[371,372,379,385] oscillations may be confined to certain experimental conditions and pathway topologies.[297,386-388] Bistability means that the system is either "off" or "on," even after the initiating stimulus has ceased. Thus, bistability is advantageous in biological processes that require irreversible decisions, such as cell cycle progression, differentiation, or cell death. In contrast, the biological role of oscillations of activities in signaling pathways is less obvious but may facilitate the temporal synchronization of processes.

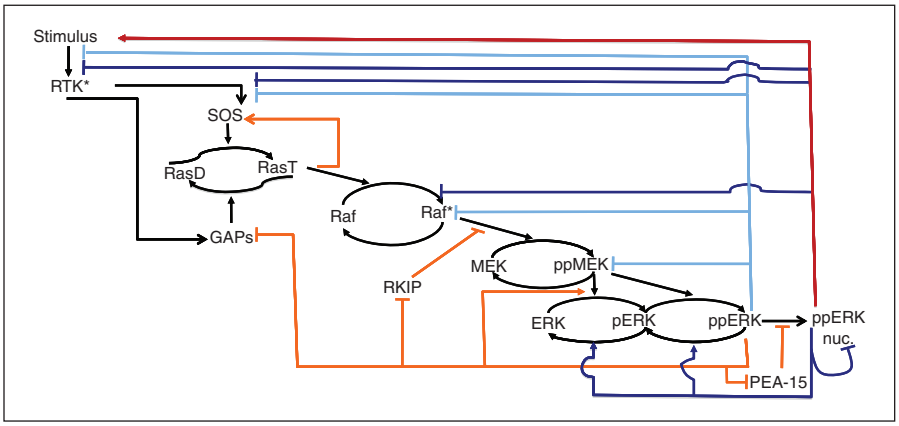

Figure 6. Schematic diagram of feedback mechanisms in the Ras-ERK pathway. Short- and long-term negative feedbacks are colored light and dark blue, respectively. Short- and long-term positive feedbacks are colored orange and red, respectively.

Feedback Mechanisms: Tuning the Dynamics and Input/Output Sensitivity

The properties arising from signal and sensitivity amplification provide advantages, for instance, the ability to respond to small input signals, while filtering out noise through the switchlike behavior. However, there are also tradeoffs. Amplifiers may overshoot the desired output levels, and the more sensitive the cascade becomes, the quicker the output will saturate, and it will respond to a smaller range of inputs. Biology has found ways to avoid these tradeoffs, and they include feedback mechanisms. Modeling is very useful in analyzing how feedback loops change the behavior of a biological system, as it is usually difficult to isolate these feedbacks in an experimental setting but easy to do so within a mathematical model. The Ras/ERK pathway features a number of negative and positive feedback loops (Fig. 6), which can generate a wide variety of dynamic behavior.

Multiple layers and roles of negative feedback. The light blue lines in Figure 6 denote short-term negative feedback loops, which begin to act nearly immediately upon activation of ERK. Activated ERK leads to the phosphorylation and inactivation of the RasGEF SOS,[46,47] and modeling suggests that several phosphorylation sites on SOS all independently mediate strong negative feedback.[389] ERK feedback phosphorylation affects targets at each upstream activation step. The first is the EGFR. Phosphorylation of T669 by ERK triggers multiple effects including decreases in receptor internalization, kinase activity, and the phosphorylation of selected substrates.[390,391] Blocking this feedback inhibition by MEK inhibitors increased EGFR activity and enhanced epithelial-to-mesenchymal transition and migration.[392] The next layer of negative feedback targets comprises adaptor protein working immediately downstream of the EGFR, that is, the Grb2-SOS complex,[46,47] and Gab1, a scaffolding protein involved in PI3K and RasGEF recruitment to the plasma membrane.[393] Further downstream, Raf-1,[92,385] B-Raf,[82,144,394] and MEK[158] are phosphorylated, leading to decreased pathway activity as discussed above.

When strong negative feedback is combined with the amplifier, constituted by the Raf-MEK-ERK cascade, the pathway adopts characteristics of a negative feedback amplifier (NFA).[385] This circuitry is widely employed in electronic systems to confer response linearization and robustness to noise.[395] These properties also exist in the biological NFA, as predicted by mathematical modeling and experimental validation.[385] First, the NFA rendered ERK activation to become more linear in response to input dose, countering the effects of multiple kinase tiers and multisite phosphorylations that amplify input/output sensitivity and cause switchlike behavior. Second, ERK activation became robust to internal perturbations; for example, when MEK activity was incompletely inhibited, the attenuation of the negative feedback permitted the input to rise and sustain MEK activity. Breaking the negative feedback, for example, by expression of Raf-1 mutants that are resistant to feedback phosphorylation by ERK, very effectively sensitized the system to MEK inhibitors. Several important

conclusions emanated from the analysis of the NFA design. Proteins embedded in NFAs are difficult drug targets because the NFA design will buffer any inhibition that is not complete. In contrast, inhibition of inputs outside of the NFA module, such as growth factor receptors, produced a linear dose response curve. Even more interestingly, weakening the negative feedback by a Raf inhibitor drastically improved the efficacy of a MEK inhibitor. Thus, the mathematical model made a concrete suggestion for a highly efficient drug combination, that is, Raf and MEK inhibitors, which from the experimentalists' point of view appears so counterintuitive that it probably never would have been tested. Thus, the analysis of design principles of signaling pathways by mathematical models can give very applicable results.

The next layer in time is the delayed negative feedbacks that emanate from ERK but that are mediated by the transcriptional induction of feedback inhibitors. They are depicted by the dark blue lines in Figure 6 and only begin to take effect approximately 30 minutes or longer after ERK activation. Active ERK induces the transcription of multiple cytoplasmic and nuclear dual-specificity phosphatase (DUSP) isoforms, which dephosphorylate and deactivate ERK.[396,397] In some instances, the stability and/or the phosphatase activity of DUSPs is also controlled by ERK activity, resulting in a positive feedforward loop embedded into this negative feedback.[398,399] In addition, ERK stimulates the transcription of other pathway inhibitors, such as the Sprouty and Spred proteins,[304,306] which inhibit EGFR endocytosis, RasGEF recruitment, and Raf activation, and Mig6/RALT, which not only inhibits the activity of various receptor tyrosine kinases (RTKs)[400,401] but also leads to increased ErbB1 receptor degradation in a manner apparently independent from the traditional ligand-stimulated pathway.[402]

Another function of negative feedback is adaptation or return of ERK activity at steady state to near prestimulus levels despite the persistence of stimulus.[403,404] Which negative feedback(s) are responsible for adaptation was the topic of many theoretical studies, but there is still no consensus as to which are the most important in general or if other mechanisms such as receptor downregulation play a role. When ERK itself is responsible for the direct negative feedback, then the system may adapt but will not exhibit perfect adaptation, where Ras and ERK activity returns exactly to prestimulus levels.[404] The perfect adaptive behavior is characteristic of an engineering design termed "integral negative feedback," where the strength of the negative feedback is proportional to the time-integrated ERK activity. Recent modeling work suggests that the long-term, transcriptional negative feedbacks might act as such integral negative feedback circuits, as mRNA responses of active ERK-dependent genes are proportional to the total time active ERK spends in the nucleus.[397] Thus, a function of the delayed negative feedback distinct from the short-term negative feedbacks may be to achieve adaptation, that is, a resetting of the system after the response has been executed. Adaptation of biochemical networks usually requires either a negative feedback that acts proportional to the input or a negative feedback combined with a positive feedforward (incoherent feedforward loop).[405,406]

Positive feedback flips the switches. The orange and red lines in Figure 6 denote short-term and long-term positive feedbacks, respectively. The short-term feedbacks include the ERK-mediated 1) phosphorylation and inactivation of RKIP,[297] a protein that inhibits Raf's ability to activate MEK; 2) phosphorylation of PEA-15, which releases ERK from this cytosolic anchor protein and allows the nuclear accumulation of ppERK[407,408]; and 3) phosphorylation of NF-1, a RasGAP whose ability to convert RasGTP back into the inactive RasGDP form is inhibited by ERK phosphorylation.[408] In addition, RasGTP produced by the RasGEF SOS can bind to another site in SOS that allosterically stimulates GEF activity.[409] In T lymphocytes, this positive feedback is key to establish a bistable Ras response that contributes to the establishment of memory cells, which maintain the ability to be rapidly reactivated by the specific antigens they have encountered before.[410] The biological relevance of positive feedback includes the generation of bistability. Bistability typically is brought about by strong positive feedback, which maintains the response even after the input was removed. Thus, bistability in the Ras/ERK pathway underlies processes that require clear and sustained "on" and "off" signals, such as memory formation in individual neurons[411] and cell fate decisions of neuronal[371,408] and lymphoid cells.[410,412] The long-term positive feedbacks involve the ERK-induced autocrine production or release of growth factors that entertain further ERK activity by stimulating receptors.[413] Such autocrine mechanisms are widely implicated in development, cell differentiation, and tumorigenesis.[414-416]

A Brief History of Crosstalk: Integrating Various Signals

Although we know that the Ras/ERK pathway is only part of a much larger network, most of our analysis treats it as an isolated entity. This approach has proven very effective, but we have to be aware that much of the distinction between pathways may simply reflect the historic sequence of discoveries that conveniently helps us to compartmentalize the network into conceptually and experimentally accessible entities. From this vantage point, we usually summarize connections between pathways as crosstalk. Much of the known crosstalk between ERK and other pathways is positive, but there are also modes of negative crosstalk. The assortment of crosstalks discussed here is certainly incomplete, and many of the crosstalk mechanisms are likely to be cell type dependent. However, a striking observation is that many crosstalk mechanisms are under the control of Ras or act on Ras, suggesting that Ras plays a major role in coordinating this crosstalk.

A prime example is PI3K, which can be activated by receptors and by Ras directly.[417] PI3K phosphorylates $PIP(4,5)_2$ to produce $PIP(3,4,5)_3$, which binds with high affinity to proteins containing pleckstrin homology (PH) domains, such as the Gab scaffolds.[418] This allows PI3K to facilitate activation of Ras through 2 mechanisms, as Gab1 recruits SOS complexes and also the phosphatase SHP2.[419] SOS directly activates Ras, while SHP2 maintains RasGTP levels by dephosphorylating residues on RTKs that recruit RasGAP.[420] Furthermore, PI3K also leads to activation of PAK, which phosphorylates Raf-1 on the activating S338 residue.[72] A main effector of PI3K is Akt, which intersects with the ERK pathway on several levels. Akt was reported to inhibit Raf-1 by phosphorylation of S259,[103] which however was not substantiated in subsequent work.[104,105] More interestingly, Akt shares several substrates with ERK, where phosphorylation by ERK and PI3K acts synergistically. Examples include the proapoptotic protein BAD, which is jointly inactivated by Akt phosphorylation and ERK-dependent phosphorylation.[421] Similarly, both Akt and ERK jointly phosphorylate PEA-15, a cytosolic anchor for ERK, thereby inducing the release of ERK and allowing ERK phosphorylation and nuclear accumulation.[408] In this case, positive crosstalk is coupled with positive feedback, which is likely to yield highly nonlinear synergistic effects. Another example for positive crosstalk emanates from the PLCγ pathway, which is activated by many RTKs on the same time scale as Ras. Active PLCγ cleaves the phospholipid $PIP(4,5)_2$ to produce diacylglycerol (DAG) that stays in the plasma membrane and soluble inositol triphosphate (IP_3) that induces a calcium release from the endoplasmic reticulum (ER). DAG and calcium, alone and in combination, can activate 2 classes of proteins important for Ras/ERK signaling: a family of RasGEFs called the RasGRPs and various protein kinase C (PKC) isoforms. There is not yet evidence of whether the RasGRPs are subjected to negative feedback regulation as is SOS. However, RasGRP primes SOS for allosteric activation by RasGTP and thereby plays an essential role to cause the bistable Ras activation in T lymphocytes discussed above.[410] However, calcium via its binding protein calmodulin also can inhibit Ras-mediated activation of ERK signaling.[422,423] Activated PKC enhances signaling through the ERK pathway through several mechanisms including the inhibition of RKIP by phosphorylation on S153.[371,424] RKIP can also be disabled by ERK phosphorylation on S99,[297] and this event plays a major role in the crosstalk between the ERK and Wnt pathways.[425]

What is the purpose of all this crosstalk? Likely, the primary reason is the integration of various environmental cues by the cell to make appropriate decisions. For instance, it is known that under normal circumstances, adherent cells will not proliferate while not attached, and the crosstalk between adhesion/PI3K signaling and ERK signaling may coordinate this response.[73,74] Similarly, the crosstalk between the cAMP and ERK systems, which was discussed above, can inhibit Raf-1 but stimulate B-Raf.[426] Thus, the specific response depends on the expression of Raf-1 and B-Raf, which can differ between cell types and tissues.

Conclusions

What have we learned about the Raf-MEK-ERK pathway as an effector of Ras in the 17 years since this relationship was brought to light? A simple summary could be that Rafs are the "first in, last out" in Ras signaling. In accounting, this is a method of inventory valuation, which is based on the assumption that the cost of goods purchased first is the cost of goods sold last. In other words, in a growing enterprise, the value of the old stock rises with the value of new additions. Undoubtedly, exploring the Ras signaling network is a blooming industry. We now have entered a phase in which the mapping of the components of signaling networks is rapidly progressing and for the Ras network may soon approach completion. However, the result is a telephone book full of names rather than an ordnance survey type of map that in detail connects the names with pathway topologies. We also need to be able to trace the wanderers' steps in time to understand the function of the pathways. Here again, the old stock is taking the lead in developing approaches to draw the missing lines, track the temporal relationships, and analyze the composite behavior of simple modules embedded in complex networks. The most important insights will be those that transcend the directory of names and provide an understanding of the design principles of biological systems and their evolution. This will enable us to address the next big challenge: How do biochemical signaling networks generate biological specificity? The current state of analysis of the Ras-Raf network offers a glimpse into this new world. The concepts and tools developed in this process hopefully will widen this glimpse into a window overlooking the whole Ras network.

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The author(s) declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

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