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Review

Back to the roots: the remarkable RAF oncogene story

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Abstract. RAF kinases entered the limelight when our understanding of the genetic nature of cancer was much less defined and the seminal importance of proto-oncogenes as components of intracellular signaling pathways was just beginning to be recognized. Following the discovery of the v-RAF oncogene and the subsequent description of the *c-RAF*-1 gene by the group of Ulf Rapp, the last 20 years have seen the dissection of the signaling pathways in which RAF kinases function, and the cellular processes they control. The recent demonstration of mutations in B-RAF and C-RAF in human tumors marked the return of RAF kinases to their roots as oncogenes. The availability of small molecular weight inhibitors has fueled the hope for new therapeutic approaches. Despite the deep insights gained through the work of many laboratories, the past has left us with sufficient controversy and plenty of open questions to keep RAF research as interesting as ever.

Keywords. RAF, signaling, human cancer, transformation, mutation.

From the viral RAF oncogene to the proto-oncogene family of RAF serine/threonine kinases

RAF was initially encountered in two different viruses, the naturally occurring avian retrovirus Mill Hill 2 (MH2), and the murine sarcoma virus (MSV) 3611 isolate discovered during experiments aiming to isolate novel transforming genes. MH2 contains the avian homologue of viral RAF (v-RAF), called v-MIL or v-MHT, together with the nuclear oncogene v-MYC, which has been shown to cooperate with RAF in various aspects of transformation [1]. 3611-MSV was recovered from a mouse which had developed histiocytic lymphoma and lung adenocarcinoma [2]. The name RAF derives from the observed enhancing effect of 3611-MSV on the fibrosarcoma induction in newborn NSF/N mice, hence rapidly accelerated fibrosarcoma, or RAF. Viral *RAF* genes originate from their cellular counterparts through homologous recombination, and in both viruses an N-terminal truncation of the cellular proto-oncogene as well as a fusion with viral gag-protein-derived sequences occurred during this process resulting in the constitutive activation of the kinase [3]. Functional characterization of v-RAF or activated versions of C-RAF either alone or in combination with other oncogenes was instrumental in dissecting the contribution of RAF to many physiological and non-physiological processes including proliferation, cell survival, differentiation and cellular transformation.
Over time, additional C-RAF homologues were identified in mammals, referred to as A- and B-RAF [4]. All RAF proteins are serine/threonine kinases and show a common architecture characterized by three conserved regions (CR1–3) of high homology. CR1 and CR2 are located in the N-terminal half of the protein, the so-called regulatory domain, whereas CR3 comprises the kinase domain. The molecular weight ranges from 68 kDa for A-RAF, to 72 kDa for C-RAF and 72–100 kDa for B-RAF, the latter being subject to alternative splicing. All RAF

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isoforms are expressed fairly ubiquitously, with varying levels [5].

RAF kinases as an integral part of intracellular signaling cascades

Through combined biochemical and genetic approaches, RAF kinases have been shown to be components of an evolutionarily conserved signaling cascade that links receptor activation at the cell membrane to the modification of cytoplasmic or nuclear targets that are required for the execution of developmental programs as well as for cell survival and proliferation [4]. This pathway, which includes RAS-RAF-MEK and ERK, is also referred to as the mitogen-activated protein kinase (MAPK) or cytoplasmic cascade.

RAF activation has been observed in response to many mitogenic stimuli, which bind and stimulate cell surface receptors with intrinsic or associated tyrosine kinase activity [4]. The small GTPase RAS functions as a critical switch molecule in relaying receptor activation to a three-tiered cascade, which in addition to RAF consists of the dual-specificity kinases MEK1,2 and their substrates ERK1,2 [6]. A multitude of nuclear but also cytoplasmic ERK targets that are important for cell fate decisions and cell-type-specific functions have been described [6]. The events leading to the activation of RAF kinases are complex and still incompletely understood (Fig. 1). Several recent reviews provide detailed insight into this topic and readers are referred to these resources for an in-depth discussion [7–10]. A simplified scenario for the activation of C-RAF includes the following sequence of events. (i) In resting cells, C-RAF adopts an inactive configuration which prevents interaction with substrates. Binding of 14-3-3 proteins to phosphorylated serines at positions 259 and 621 helps to lock the enzyme in this dormant state. 14-3-3 proteins are members of an evolutionarily conserved family of cytoplasmic proteins, which interact with other proteins via a phosphoserine/phosphothreonine-containing recognition motif [11]. (ii) Growth factor binding results in receptor tyrosine kinase (RTK) activation and phosphorylated RTK tyrosine residues help to recruit guanine nucleotide exchange factors (GEFs), which promote the transition of the GDP-bound inactive form of RAS to active RAS-GTP [12]. RAS proteins predominantly reside at the inner side of the cell membrane, where they provide a platform for the recruitment of RAF kinases [13]. RAF kinases physically interact with RAS and in addition with membrane lipids through the RAS-binding domain (RBD) and a cysteine-rich domain (CRD) adjacent to the RBD in CR1. This membrane localization provides the prerequisite for the subsequent full activation of the kinase through (iii) phosphorylation of serine and tyrosine residues. In the case of C-RAF, this phosphorylation may involve p21-activated kinase (PAK) and SRC family kinases [7–10]. As a consequence, the regulatory domain dislocates from the kinase domain allowing substrate access. Hetero- and homodimerization of RAF proteins also may play an important regulatory role in the process of RAF activation. Artificially induced oligomerization of C-RAF proteins resulted in their activation [14, 15] and C-RAF and B-RAF can form heterodimers following RAS activation [16]. A small subset of mutant B-RAF proteins found in human cancer (see below) lacked kinase activity but nevertheless activated C-RAF, which then signaled to MEK [17]. C-RAF activation required 14-3-3-dependent heterodimerization of C-RAF and B-RAF, and transphosphorylation. This activation mechanism also applied to wild-type B-RAF, but complex formation in this case was RAS induced [18]. Once activated, RAF kinases transmit the signal to downstream MEK1,2 kinases, which in turn phosphorylate and activate ERK1,2. MEK1,2 remain the best-characterized targets for RAF and in many instances their requirement in the downstream transmission of RAF signals has been demonstrated [19, 20].

Intracellular signaling is commonly seen as a process which originates at the cell membrane to modify cytoplasmic or nuclear targets. However, evidence has been accumulating for the existence of intracellular platforms, where MAPK signaling originates or where activated pathway components operate. Apoptosis suppression by a truncated and constitutively active form of C-RAF, RAF-BXB [21], has been shown to require translocation of the kinase to the mitochondria [22]. Mitochondrial localization was achieved by overexpressing Bcl-2, which binds C-RAF and resides at the outer mitochondrial membrane, or by providing RAF-BXB with a mitochondrial-targeting sequence. More recent work suggested that mitochondrial RAF may function in a physiological setting. Basic fibroblast growth factor (bFGF) protected cells against intrinsic stress stimuli through a mechanism which involved serine 338/339 phosphorylation of C-RAF as well as mitochondrial translocation [23]. Protection against activators of the extrinsic cell death pathways by vascular endothelial growth factor (VEGF), on the contrary, occurred following phosphorylation of tyrosines 340/341 and proceeded through the C-RAF effector MEK [23]. Phosphorylation of serines 338/339 by PAK1 can be the initiating signal for mitochondrial translocation of C-RAF, which leads to the phosphorylation-inactivation of the pro-apoptotic Bcl-2 family member BAD [24]. While it seems intriguing that C-RAF regulates cell survival at a site in the cell which is essential for the control of energy production and cell survival [25], the critical targets for mitochondrial RAF as well as the regulation of its activity and localization at this site remain to be defined.

More recently, additional intracellular sites for MAPK signaling have been suggested, one of them being the en-

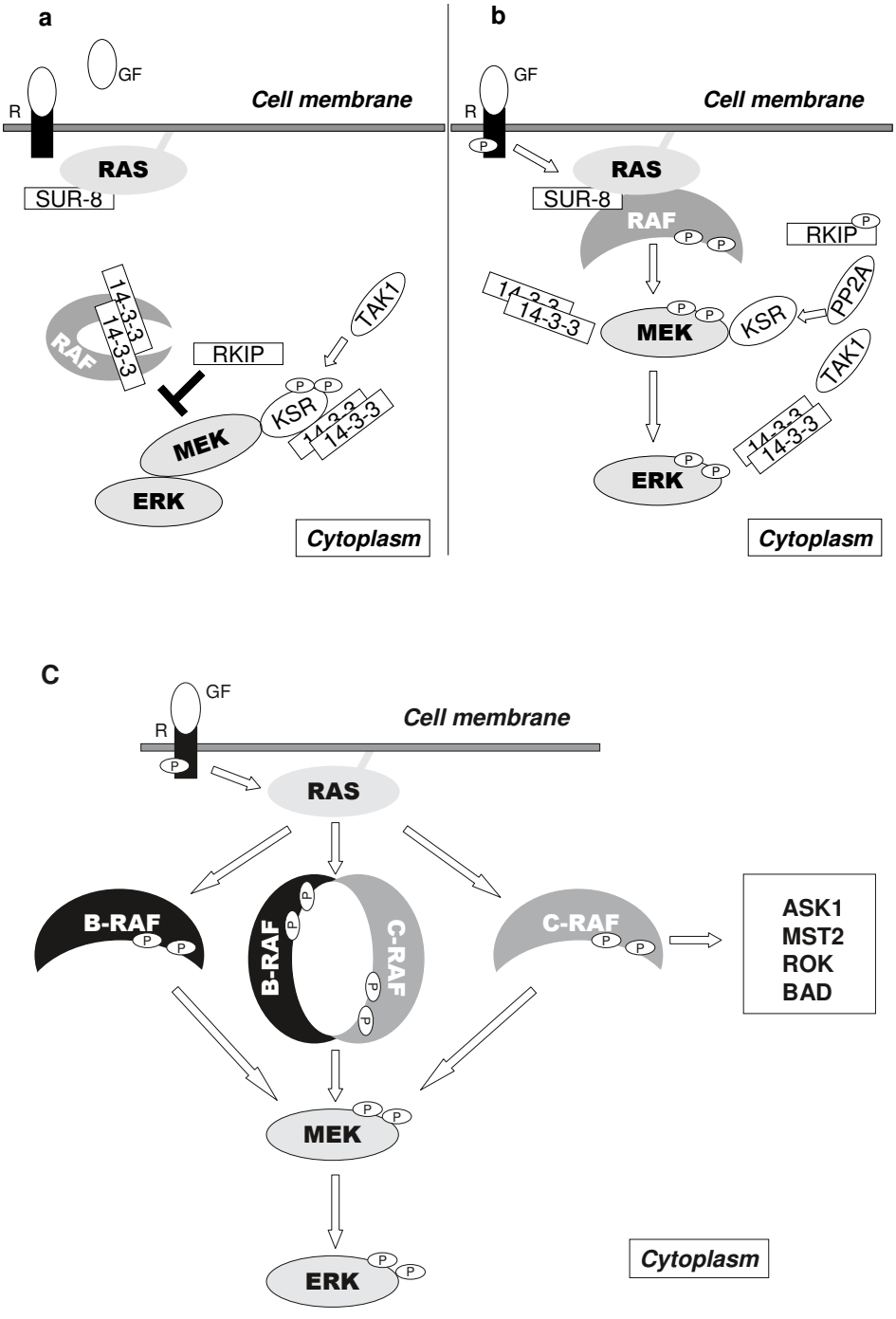

Figure 1. Association of C-RAF with other signaling molecules during the activation process. A detailed description of these processes is given in the text. GF, growth factor; R, receptor. (*a*) The situation in the resting cell. (*b*) The activated RAF signaling complex at the cell membrane. (*c*) Possible cross-talk between different RAF isoforms in the activation of MEK as well as MEK-independent RAF effectors.

dosome. Activation of RTKs through ligand binding is followed by internalization of the receptor – most likely together with components of the MAPK pathway – via clathrin-coated vesicles (CCVs), which fuse with the endosome [26]. During the trafficking through the endosomal compartment, activated receptors are sorted for degradation, whereas inactive ones are recycled back. Receptor signaling is maintained during endocytosis [27]. The MAPK cascade can localize to this compartment through the adaptor protein p14, which in turn binds the MEK1/ERK1 scaffold protein MP-1 [27]. Signaling from components of the cytoplasmic cascade has also been demonstrated for intracellular compartments that are further removed from the plasma membrane, namely the Golgi and the endoplasmic reticulum (ER). The pathway regulating RAS activation at the Golgi also initiates at the

plasma membrane through growth factor of inulation, but differs from the classical scheme for RAS activation and shows a delayed response but sustained signaling [28]. C-RAF itself has not been associated with the Golgi; however, RAS targeted to this location activated ERK and transformed fibroblasts [29] suggesting that the intermediate components in this pathways, RAF and MEK, are also active at this site. We do not yet know which fractions of RAF distribute to various cellular compartments and whether signaling from the same molecule at different cellular sites will have different outcomes in terms of cell survival, proliferation, differentiation or transformation. Understanding these differences will have important implications for therapeutic approaches aiming at the inhibition of RAF kinases.

The core elements, RAS/RAF/MEK/ERK, are sufficient to account in theory for the signal propagation downstream of a cell membrane receptor to nuclear and cytoplasmic targets. However, in living cells, the RAF proteins with molecular weights ranging from 68 to 100 kDa have been proposed to be part of a much larger, 300- to 500-kDa complex. Biochemical and genetic approaches have defined more than 30 RAF-interacting proteins, which assist in signal propagation by facilitating the assembly of signaling proteins or orchestrating them at different subcellular sites [8]. Regulators acting at the level of RAF include, among others, RKIP, KSR, 14-3-3, CNK1, and SUR-8 (Fig. 1). KSR (kinase suppressor of RAS) was identified in genetic screens as a positive regulator of RAS signaling. Despite homology to serine/threonine kinases, replacement of a highly conserved lysine required for the phosphotransfer reactions results in the loss of its kinase activity. KSR thus most likely functions as a scaffold protein interacting, among others, with MEK and 14-3-3 in its resting state and thereby assuring signal propagation along the components bound [30]. 14-3-3 binding following phosphorylation by TAK1 (TGF-β-activating kinase) keeps KSR, and thus also MEK, in the cytoplasm. In mammalian cells, the interaction of KSR with C-RAF is RAS dependent. Following growth factor stimulation, PP2A dephosphorylates KSR and thereby causes 14-3-3 displacement. As a consequence, the KSR-MEK complex can move closer to RAF, which at this time is already activated at the plasma membrane. CNK (connector enhancer of KSR) and suppressor of RAS-8 (SUR-8), are additional modulators of RAF signaling. Both were discovered during genetic screens in *Drosophila* and *Caenorhabditis elegans* and directly interact with C-RAF [30]. SUR-8 is found in a complex with RAS and C-RAF in mammalian cells and it enhances RAS signaling by fostering RAS-RAF interaction. CNK interacts with the C-terminal kinase domain of RAF and facilitates assembly with signaling complexes at the plasma membrane, which further supports full RAF activation. RAF kinase inhibitor protein (RKIP) was initially isolated as a C-RAF-interaction protein, which also binds MEK and ERK [31, 32]. However, RKIP does not function as a scaffold for these kinases, but, rather, interferes with the binding of RAF to MEK and thereby prevents phosphorylation and activation of MEK. During mitogen stimulation, RKIP becomes phosphorylated and dissociates from RAF to allow for downstream signaling. [33]

Evolution of RAF kinases

Homologues of C-RAF have been identified in several other species, with a single gene present in *Drosophila melanogaster* (*D-RAF*), *C. elegans* (*lin-45*) and plants (*CTR1*). These genetic systems of reduced complexity helped to establish non-redundant functions of RAF in developmental pathways, and to order signaling molecules within pathways. This analysis confirmed that components and the makeup of this pathway have been maintained throughout evolution. Despite the use of alternative names for most of the proteins involved they are true homologues of their mammalian counterparts.

Drosophila melanogaster

Drosophila RAF (D-RAF) is more closely related to B-RAF than to A- or C-RAF. B-RAF therefore may be the functional homologue of the ancestral RAF proteins of *D. melanogaster* and *C. elegans* [34]. The RTK-RAS1-DRAF-DSOR(MEK)-Rolled (MAPK) cascade is involved in several developmental pathways during embryonic development and has been studied extensively for its involvement downstream of the two RTKs, *Drosophila* epidermal growth factor (EGF) receptor (DER) and SEVENLESS (SEV). DER is required for the formation of many of the adult structures including wings, eyes, and legs, whereas SEV signaling is only essential during *Drosophila* eye development. Eight hundred single eye units (ommatidia), each consisting of eight photoreceptors (R1–R8), the four lens-secreting cone cells, and accessory cells are generated during larval development in a strictly orchestrated sequence from a monolayer epithelium, the eye-antennal imaginal disc. In a first step, R8 is specified and in a further phase, addition of the other photoreceptor cells and subsequently of the four cone cells to the growing ommatidium is performed, before the incorporation of pigment cells finally optically isolates each ommatidium from its neighbor. R7 is the last of the photoreceptor cells to differentiate, in a process which requires the binding of the ligand Bride of Sevenless (BOSS) present on the R8 cell to the SEV receptor on R7. Thus, eye development completely fails in the absence of DER-derived signals, whereas only R7 differentiation is affected by the absence of SEV. The R7 precursor cell in this case develops into

a non-neuronal cone cell [35]. Signal propagation from both RTKs proceeds in an analogues fashion as described for mammalian cells. Ligand binding results in receptor activation and in tyrosine phosphorylation, which allows for binding of the GEF Son of Sevenless (SOS) that in turn activates RAS1. Further signal propagation proceeds via D-RAF and the homologues of MEK and MAPK, D-SOR and Rolled (RL), respectively [36]. In addition, DER signaling via the MAPK pathway also has been shown to regulate cell survival during eye development [37].

Caenorhabditis elegans

The RTK-RAS (LET-60), RAF (LIN-45), MEK-2 (MEK) and SUR-1/MPK-1 (MAPK) signaling pathway in the nematode *C. elegans* has been analyzed most extensively during the formation of the vulva, a specialized epithelial structure used for egg laying [38]. The vulva forms during larval development out of six vulva precursor cells (VPCs). The three central VPCs undergo three rounds of mitosis and the resulting cells finally make up the vulva. This vulva formation is initiated through an inductive signal, which is present in all non-male larvae, and which is transmitted through the LET-60-LIN-45-MEK-SUR-1/MPK-1 cascade. Hyperactivation of this pathway has been linked to a multivulva phenotype, whereas inactivation of a gene in this cascade causes a pathologic vulvaless phenotype. Additional cell fates regulated by this cascade include the differentiation of the excretory cell, required for larval viability, and the progression of germ cells through the pachytene stage, necessary for fertility.

Plants

MAPK pathway signaling components are also conserved in plants. Ethylene is an important gaseous signaling molecule produced in response to environmental and endogenous cues, which regulates diverse metabolic and developmental processes ranging from seed germination to organ senescence. Of economic importance is its role as inducer of fruit ripening. Binding of ethylene to receptors with homology to two-component receptors found in *Escherichia coli* and *Saccharomyces cerevisiae* [39] triggers activation of ethylene-responsive genes. This cascade also includes the C-RAF-like kinase CTR1 which has been placed downstream of the receptor through epistasis experiments. Small GTPases of the RAS subfamily are strikingly absent from the sequenced genomes of plants. This goes along with the lack of upstream RTKs, which normally signal through RAS [40]. Of the multiple MEKs and MAPKs which exist in plants, none has been convincingly implicated in the ethylene response. Analysis of CTR1 function suggested a role as negative regulator of ethylene signaling. In the presence of air, CTR1 is in an active state and represses an ethylene response. In the presence of ethylene, a conformational change may be induced, relieving this repression [41, 42].

RAF functions in mammals

RAF kinase functions in mammals have been analyzed in great detail through combined biochemical and genetic approaches. Following the isolation of v-RAF, the oncogenic function of this kinase family was a strong focus of research, despite a lack of evidence for an involvement in human tumor development at this time. Activated versions of A- and B-RAF kinases generated in analoguous fashion to the retroviral v-RAF protein shared the ability to transform established fibroblast cell lines [3]. This oncogenic potential also extends to naturally occurring mutants of B-RAF and C-RAF, which have been isolated recently [43, 44]. Transformation has also been observed *in vitro* for constitutively active versions of MEK [19] and ERK [45], suggesting that these effectors are critical for the propagation of signals required for transformation. Moreover, many upstream components present in a mutated form in tumors (RTK, RAS) activate this cascade and depend on it for transformation [20]. Cross-talk with other signaling entities has also been observed in the case of RAF, and involvement of autocrine mechanisms has been suggested in some instances. They include the activation of NF-κB [46], PI3-kinase/PKB [47, 48] and also of stress kinases [49]. In all cases, the contribution of these recruited pathways to the transformation in an *in vivo* setting has not been analyzed in detail. However, this activation of multiple pathways may be characteristic for malignant diseases, where genetic aberrations are usually abundant [50] and thus several intracellular signaling pathways will be affected.

In the following, we will address mechanisms, which have been implicated in RAF transformation and the effector pathways involved.

Cell cycle progression. Cell cycle progression is critically regulated through the action of kinases and essential cofactors. Cell cycle entry depends on the presence of extrinsic factors to proceed factor independently to enter S phase and complete the cell cycle beyond the so-called restriction point. Cell cycle progression is regulated through cyclin-dependent kinases (CDKs) and their regulatory subunits, the cyclins. Mitogen stimulation requires CDK4 and 6 and the interacting D-type cyclins. D-type cyclins are unstable and their synthesis and assembly with CDKs is growth factor regulated. CDK activation results in the phosphorylation of the retinoblastoma (Rb) protein, and thereby relieves its suppressive action on the transcription of genes required for DNA synthesis [51]. Activation of the RAS-RAF-MEK-ERK pathway has been shown to stimulate cell cycle progression, mainly through transcriptional upregulation of cyclin D1. Ad-

ditional cell cycle targets for RAF signaling include the CDK inhibitors p27 and p21. p27 protein levels are decreased in cells expressing constitutively active mutants of RAF by a mechanism which may lead to enforced p27 degradation. Analysis of signaling through this cascade also highlighted the importance of signaling strength. Hyperactivation was linked to the induction of growth arrest. The molecular basis for this observation may lie in the high levels of p21 expression caused by strong signals leading to growth arrest, whereas low levels of p21 are permissive for proliferation [52–54].

Cell survival. Cell survival is critically regulated by the major intracellular signaling pathways. The demise of a cell is initiated through the activation of proteolytic enzymes (caspases) by death-receptor-dependent (extrinsic) and on strictly mitochondria-dependent (intrinsic) pathways [55]. Multiple safeguard mechanisms are in place which normally prevent caspase activation or inhibit already activated caspases [56–59]. The survival activity of RAF was first demonstrated for the oncogenic form of the kinase [60]. Expression of gag-v-RAF delayed the apoptotic cell death of interleukin (IL-3)-dependent pro-myeloid 32D cells upon growth factor withdrawal, but was by itself like other pro-survival proteins insufficient to prevent cell death for prolonged periods of time. A role for C-RAF in cell survival signaling has also been supported by the increased apoptosis observed in RAF-deficient animals and cells [61–63]. Recent research has dissected the pathways and the mechanisms through which RAF controls cell survival. It has demonstrated the existence of several RAF effectors involved in this process, but has also provided evidence for direct C-RAF effects, mainly mediated through its interaction with critical regulators of apoptosis [56]. Where analyzed, protection by RAF usually resulted in the maintenance of mitochondrial integrity. In a single instance, a protective effect through B-RAF following cytochrome c release has also been reported [64]. Based on the analysis of B-RAF-deficient motoneurons, upregulation of inhibitor of apoptosis proteins (IAPs), which can bind and inhibit active caspases, may be one mechanism operating at the post-mitochondrial level [65]. Inactivation of the pro-apoptotic Bcl-2 family member BAD has been reported as one possible mechanism through which RAF kinase assures cell survival, although how commonly BAD is involved in cell death induction remains to be determined [66]. Other direct mechanisms for anti-apoptotic RAF signaling were suggested by the interaction of C-RAF with the apoptosis-inducing kinases MST-2 [67] and ASK1 [68], resulting in the inhibition of their pro-apoptotic activity.

Controversy exists around the issue of MEK requirement in survival signaling by RAF. While *in vitro* studies clearly demonstrated an essential role for MEK in survival signaling downstream of RAF [48], the knock in of a mutant of C-RAF to the C-RAF locus, which failed to activate MEK, was indistinguishable from wild-type C-RAF with regard to cell survival [61], arguing that signaling via MEK and ERK is not required for apoptosis suppression by C-RAF. The controversy stems from previously published data [69–71], which suggested that the mutations used failed to completely inactivate the kinase and thus residual signaling through this cascade may persist. True kinase-dead mutants of C-RAF, however, were as efficient as wild-type RAF in interacting with MST2 and preventing apoptosis [72]. Survival through MEK and ERK contributes to apoptosis suppression through the inactivation of pro-apoptotic BH3-only members of the Bcl-2 family [73], the transcriptional upregulation of IAPs [65] and the activation of protein kinase B (PKB) via autocrine mechanisms [47, 48].

Major anti-apoptotic pathways not only prevent caspase activation or activity but also indirectly contribute to cell survival by regulating cellular energy production and use. Different mechanisms for achieving this goal have been documented previously [25, 74] and even in the case of RAF, a linkage to cellular energy production has been suggested. Extra-mitochondrial energy production is critically affected by A-RAF [75], and its mitochondrial counterpart may be susceptible to regulation by C-RAF, as suggested by our own data [A. Garedew, C. Doblander, E. Gnaiger, J. Troppmair, unpublished data].

RAF-dependent signaling pathways are also able to prevent excessive levels of reactive oxygen species (ROS) and calcium concentrations in this organelle [A. Kuznetsov, C. Doblander, M. Janakiraman, M. Hermann, M. Wurm, R. Sucher and J. Troppmair, unpublished data], which directly induce cell death. From these and other data it seems realistic to postulate that RAF signaling controls mitochondrial events, however, the existence of intermediates in these pathways and the nature of mitochondrial targets remain to be demonstrated. In this context, the recent demonstration of a prohibitin-C-RAF complex required for RAS-induced MEK/ERK activation may be of particular interest [76]. Prohibitins are evolutionarily highly conserved and ubiquitously expressed proteins with proposed functions as tumor suppressors, regulators of apoptosis and mitochondrial function. Mitochondria may be the main site of prohibitin function and localization in the cell, where they assist as chaperones in the assembly of mitochondrial respiratory chain complexes [77]. Whether C-RAF can be found associated with mitochondrial prohibitin and whether such an interaction provides access for C-RAF to mitochondrial substrates remains to be shown.

Cell migration. Cell migration is an essential part of embryonic development, but is also required for wound healing, during angiogenesis, and in tumor metastasis. Cyto-

skeletal reorganization is a prerequisite for cell mobility and the small GTPases of the Rho family are key regulators of this process [78]. Rho induces actin reorganization via the effectors Rho-kinase/ROK/ROCK and mDia [79]. Conditional ablation of C-RAF in keratinocytes has shown recently that C-RAF is required for efficient wound healing [80]. RAF deficiency did not affect keratinocyte cell survival or proliferation but rather severely impaired their migration. ROK was shown to physically interact with C-RAF and deletion of C-RAF resulted in ROK hyperactivation and mislocation and thus deregulation of Rho downstream signaling [80]. Surprisingly, this defect could also be reversed through the expression of a kinase-dead mutant of RAF, suggesting functions of RAF which are independent of MEK/ERK activation. In contrast, increased migration was observed in B-RAF-deficient cells through a process which also targeted Rho signaling but was MEK/ERK dependent [81].

Developmental effects. Developmental effects of RAF kinases have been studied through gene knockouts – at the organism level for all three RAF isoforms and at the organ level for C-RAF. Intestinal and neurological abnormalities have been observed in the case of A-RAF, and depending on the genetic background, animals died from extensive bowel distension 7–21 days postpartum (C57/Bl/6). Even survivors without intestinal abnormalities showed neurological defects (129/OLA) [82]. In an initial C-RAF knockout, the first coding exon was replaced by a drug resistance gene, resulting in the generation of a 62-kDa N-terminally truncated protein with reduced activity. Embryos homologous for the altered allele grew slowly and died by E12.5, most likely due to impaired placenta function. However, on an outbred CD1 background, two-thirds of the mutant mice survived until birth and died thereafter of failed lung maturation. A complete knockout of C-RAF was achieved by targeting exon 3 resulting in a complete loss of C-RAF protein expression [62]. Again, the embryos showed growth retardation and placental anomalies. The fetal livers were hypocellular and contained numerous apoptotic cells. B-RAF-deficient mice generated through the partial deletion of the RAS-binding domain died *in utero* (E10–E12.5). Development of the vasculature was disturbed and apoptosis was observed throughout the embryo and, most significantly, affected the vascular endothelium [83].

RAF kinases as oncogenes in humans

B-RAF mutations

While much of the research on RAF kinases for a long time focused on C-RAF, it gradually became clear that B-RAF may be even more critical for the transmission of mitogenic signals to the MEK/ERK module. The general interest in B-RAF was further spurred when, in June 2002, activating mutations of B-RAF were described in 66% of melanomas and at a lower frequency in a wide range of human solid cancers [43]. All mutations were located within the kinase domain of B-RAF with a single substitution (V600E, formerly V599E [5]) accounting for 80% of them (Fig. 2). Since then, more than 60 different mutations have been identified in various tumor entities. The highest frequency of B-RAF mutations is found in skin tumors (44%, with even higher frequencies in the melanoma and nevi subgroups), thyroid carcinoma [27%, again with higher prevalence in papillary thyroid carcinomas (PTCs)], ovary carcinoma (16%), large intestinal colon carcinomas (15%), and carcinomas of the biliary tract (15%). Most of the currently known B-RAF mutations are located in exon 11 or 15, within the catalytic domain. Many B-RAF mutations result in elevated kinase activity as measured in *in vitro* kinase assays or the activation of previously identified RAF targets such as NF-κB [84]. Furthermore, expression of mutant B-RAF proteins in cell lines (i.e. Cos7 or NIH3T3) [85] or *Xenopus* embryos [17] induced constitutive phosphorylation of endogenous and/or cotransfected MEK1/2 and ERK1/2. Interestingly, some mutant B-RAF proteins displayed decreased B-RAF kinase activity *in vitro* but still induced constitutive ERK phosphorylation *in vivo*. These B-RAF mutants activate endogenous C-RAF, possibly via an allosteric or transphosphorylation mechanism, thereby causing constitutive ERK phosphorylation [17]. Another hallmark of many B-RAF mutants is their ability to cause cellular transformation, as evidenced by morphological alterations in NIH3T3 cells [84], enforced cell proliferation and/or cell growth, growth in soft agar, lowered growth factor requirement [84] or tumorigenicity in nude mice [43, 86]. Furthermore, thyroid-specific expression of B-RAF V600E from a transgene resulted in PTCs that underwent dedifferentiation [87]. A causal role for B-RAF V600E could be corroborated through inhibition or depletion of the kinase, which abrogated ERK activity, proliferation and transformation of the cell lines studied, and further enhanced apoptosis [88, 89].

Further dissection of the effects of B-RAF V600E on apoptosis yielded differential results. Apoptosis suppression was abolished in B-RAF-V600E-positive human melanoma cell lines following treatment with the RAF kinase inhibitor BAY 43-9006 [90]. Contrary to this, in PCCL3 thyroid cells expressing mutated B-RAF, the increased proliferative capacity was not accompanied by net growth, due to a concomitant increase in apoptosis [91]. The authors argued that the B-RAF V600E mutation facilitates the acquisition of secondary genetic events through induction of genetic instability, which may account for its aggressive properties [91]. In a recent study, Michaloglou and colleagues [92] demonstrated that sustained B-RAF V600E expression in

human melanocytes induces cell cycle arrest, which is accompanied by the induction of p16INK4a and acidic β-galactosidase activity, both markers of senescence. Oncogenic mutations may thus induce a genuine protective physiological process like senescence, which could explain how melanocytic nevi, which harbor B-RAF V600E in more than 70% of cases, can stay in a quiescent state for decades without progressing into a malignant melanoma.

Many studies have addressed the nature of cooperating events in B-RAF transformation in human tumors. B-RAF mutations with a few exceptions do not coincide with mutations in RAS, suggesting that activations of B-RAF or RAS are equivalent in their tumorigenic effects and therefore mutually exclusive [93–97]. A further example of mutual exclusivity can be observed between B-RAF mutations and the RET/PTC rearrangement in PTCs. While each alteration alone is quite frequent in this

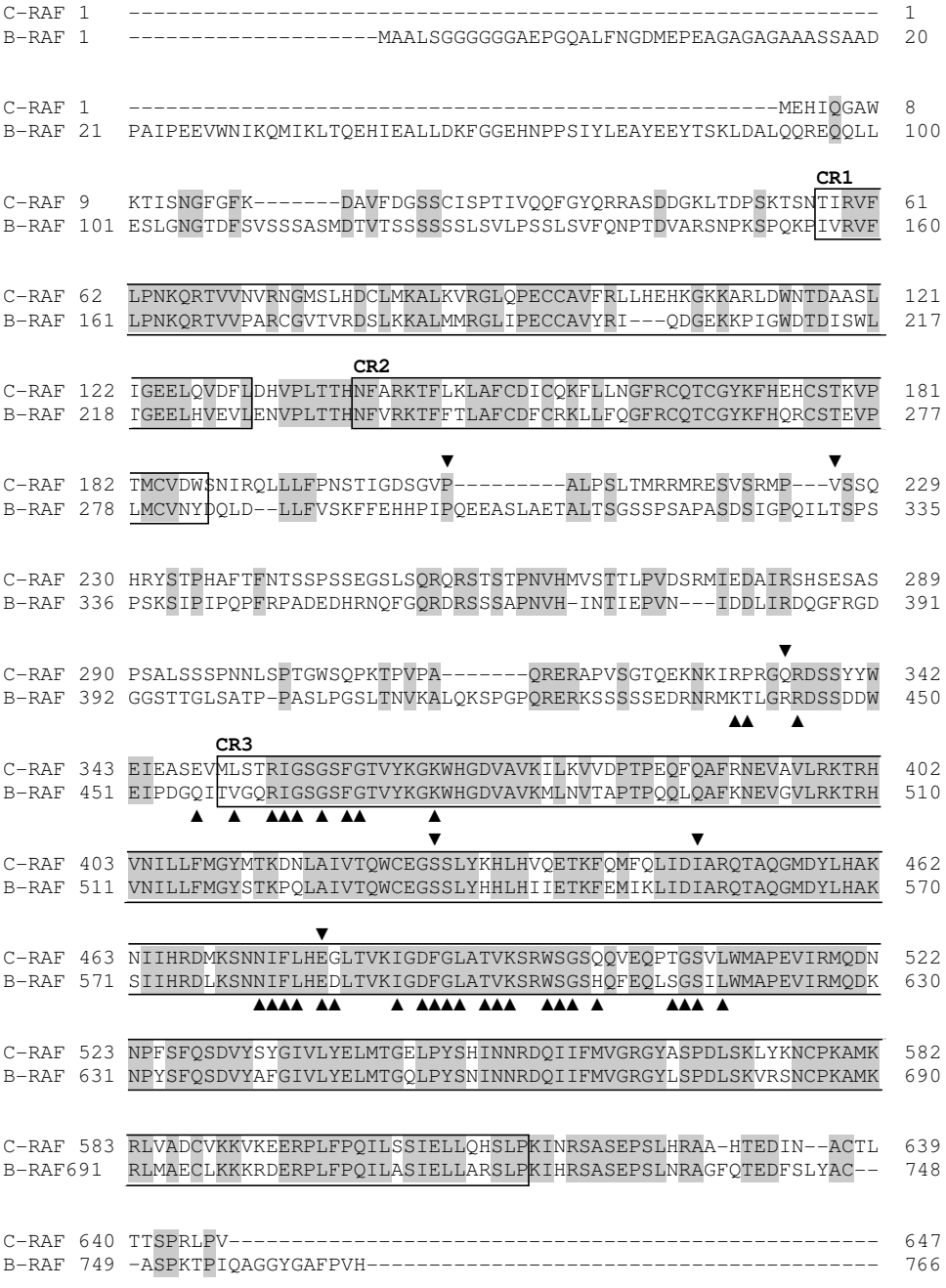

Figure 2. Sequence alignment of B-RAF and C-RAF proteins. Areas highlighted in gray indicate amino acid identity between B-RAF and C-RAF. The three conserved regions (CR1–3) are marked by frames. ▲ indicates positions in B-RAF and ▼ indicates positions in C-RAF where naturally occurring mutations have been reported.

tumor entity, they could not be detected together, again suggesting equivalent effects on PTC development [98, 99]. In one single study performed by Xu and colleagues [100], a RET/PTC-B-RAF V600E overlap could be observed – although this study has been criticized for the use of antibodies, which may not reliably discriminate between the rearranged and the wild-type RET proteins [101]. Inactivating p53 mutations have been detected in melanoma samples harboring the B-RAF V600E substitution [102]. In a transgenic zebrafish, cooperation of activating B-RAF mutations with p53 loss could be confirmed [103]. While B-RAF in all animals caused rapidly developing melanocytic nevi, only those with additional p53 deficiency progressed to invasive melanomas [103]. A critical role for p53 in this process is also supported by data demonstrating that p53 inactivation was required in B-RAF-mutated nevi to overcome the senescent state [92]. B-RAF mutations are also often coupled to the inactivation of PTEN/MMAC1 [104] or low expression of the tumor suppressor gene SLC5A8 in PTC [105]. The requirement for additional genetic alterations is also shown by the frequent linkage of B-RAF mutations to a defective mismatch repair (MMR) status [93], which is a hallmark of several human malignancies [106, 107]. However, they do not seem to be a consequence of defective MMR per se, as they are rare in the MMR-deficient subgroup with germline mutations in hMHL1 or hMSH2. Supporting this hypothesis, the vast majority of B-RAF mutations are detected in microsatellite instable cases harboring an epigenetic inactivation of hMLH1 [108].

C-RAF mutations

As described above C-RAF is the most intensively studied of the RAF isoforms. Nevertheless, the list of reported C-RAF mutations is quite short when compared with B-RAF. In 1993, the first mutations were described in a mouse model for chemically induced lung cancer [109]. All of these were consistent point mutations within a small region of the observed kinase domain and, indeed, some of them were found to be weakly transforming when tested in NIH3T3 assays. Another four exonic mutations (P207S, V226I, Q335H and E478K) were detected in four human cancer cell lines recently [110]. Although none of them resulted in the transformation of NIH3T3 cells, the E478K mutant displayed increased C-RAF kinase activity, its basal kinase activity being 25-fold higher than that of wild-type C-RAF. In an ongoing study, our group has observed the first C-RAF mutations in a human malignancy [44] (Fig. 2). S427G and I448V, both located in the kinase domain, were detected in two patients with therapy-related acute myeloid leukemia (t-AML), which occurs after chemo- and/or radiotherapy for a primary malignancy. As both mutations were absent in 200 healthy individuals (corresponding to 400 alleles), a common polymorphism could be excluded. In *in vitro* and *in vivo* kinase assays, only one mutation (S427G) resulted in increased C-RAF kinase activity whereas the other (I448V) did not. However, further experiments demonstrated that both mutants, despite their difference with respect to MEK and ERK activation, result in weak oncogenic transformation as well as inhibition of apoptosis. An additional interesting finding was that contrary to the somatic B-RAF mutations, these C-RAF mutations were of germline origin. However, as constitutive activation of the pathway was only detected in neoplastic tissues, they might constitute a hereditary predisposition to solid neoplasms and t-AML. Such genetic predisposition has been proposed particularly for patients with t-AML, considering that only a minority of individuals receiving chemo- and/or radiotherapy for a primary disease develop this type of leukemia [111].

RAF overexpression in tumors

The oncogenic potential of overexpressing wild-type C-RAF has been demonstrated following lung-targeted expression [112], although long latencies suggest the requirement for cooperating events. C-RAF overexpression in human malignancies was first described in an analysis of 27 cases of AML, where increased mRNA levels were observed in two cases with erythroleukemia [113]. This C-RAF overexpression was not confirmed at the protein level and no data about the phosphorylation status or about functional alterations are available. High expression of C-RAF mRNA could also be detected in squamous cell carcinomas of the head and neck, which could be linked to radiotherapy resistance [114]. One study observed a striking correlation between high C-RAF expression and poor survival in patients with ovarian cancer. A reduction of C-RAF protein levels and a concomitant inhibition of cell proliferation *in vitro* was seen after incubation with C-RAF antisense oligodeoxynucleotides (i.e. ISIS 5132) [115]. However, definitive data about the occurrence or the functional consequences in most human tumors are still missing, which is remarkable given that ISIS 5132 is already in clinical phase II trials.

Other RAF alterations

An interesting new way of B-RAF activation was recently described by Ciampi and colleagues [116], who analyzed B-RAF in thyroid cancer [116]. They reported a rearrangement of B-RAF via paracentric inversion of chromosome 7q resulting in an in-frame fusion between exons 1–8 of the AKAP9 gene and exons 9–18 of B-RAF. The fusion protein contains the protein kinase domain of B-RAF but lacks its autoinhibitory N-terminal portion. Hence, this rearrangement results in a constitutive, oncogenic activation of B-RAF [116]. Amplification of the

mutated B-RAF allele was observed by Maldonado and colleagues [117] in seven out of nine melanoma samples with B-RAF mutations and a gain of chromosome 7q. They therefore suggested that B-RAF mutations are one of the factors that drive selection for the frequent gain of this chromosomal region in melanoma. Loss of heterozygosity (LOH) affecting the C-RAF locus was observed in cervical cancer [118] and renal tumors [119]. However, whether these chromosomal losses have any functional consequences is not clear. C-RAF amplification has been observed in a human osteosarcoma specimen [120] and samples of urinary bladder cancer [121], but the relevance for the transformation process remains to be defined.

Targeting RAF signaling for therapeutic purposes

Due to the central role of the RAS-RAF-MEK-ERK pathway in cellular transformation, the search for clinically useful intervention strategies has been ongoing at every level of the cascade. One prime target was the small G protein RAS, which is frequently affected in many human tumors. In addition, RAF and MEK were considered targets, even before mutational activation of RAF had been reported in human tumors, based on the assumption that activating upstream events cause constitutive signaling through this pathway. Approaches chosen targeted the expression, the catalytic activity or post-transcriptional/translational modifications required for proper localization and function of these signaling molecules. Targeting RAS via farnesyltransferase inhibitors like Zanestra (R115777; Johnson & Johnson, Titusville, N. J.) or Sarasar (SCH66336; Schering-Plough, Kenilworth, N. J.) is the most established approach. Nevertheless, results obtained in clinical trials were disappointing and their efficacy could not be correlated with the presence of RAS mutations, casting doubt on whether RAS is the exclusive target for these substances [122].

RAF antisense oligonucleotides

RAF antisense oligonucleotides (AONs) induce the RNaseH-mediated degradation of RAF mRNA. Two different drugs have been tested in clinical trials so far: ISIS 5132 (CGP 69846A; ISIS Pharmaceuticals, Carlsbad, Calif.) and LErafAON (NeoPharm, Lake Forest, Ill.). ISIS 5132 is a synthetic 20-base phosphoriate antisense oligodeoxynucleotide targeted against the 3′-untranslated region of C-RAF mRNA. Promising results were obtained in preclinical studies where ISIS 5132 was shown to exhibit antiproliferative activity in cell culture and antitumor activity in animals [123]. In July 1999, the first phase I trial of ISIS 5132 was published by Stevenson and colleagues [124] and in the following 2 years, a few others followed (Table 1) [123, 125, 126]. Although the number of patients enrolled was too small for statistical evaluation, some patients showed prolonged stabilization of their disease and one patient with ovarian carcinoma had a significant response with a 97% reduction in CA-125 levels [126]. Additionally, a significant decrease in C-RAF expression levels could be correlated with administration of ISIS 5132 at doses of ≥2.5 mg/kg per day [124, 127]. However, results obtained in phase II trials were disappointing [128–131]. Only a few prolonged disease stabilizations and no single complete or partial response were observed in trials performed in lung, colorectal, prostate and ovarian cancer (Table 1). As a consequence, further clinical development of ISIS 5132 has been discontinued.

LErafAON is a new liposome-entrapped RAF antisense oligodeoxyribonucleotide with a significantly improved cellular uptake, and stable plasma levels for up to 24 h in human cancer patients [132]. Preclinical studies in nude mice bearing PC-3 human prostate cancer xenografts revealed a significant antitumor activity [133]. As experienced with ISIS 5132, the clinical development is less encouraging than the preclinical stage. No objective response could be observed in a phase I trial conducted by Rudin and colleagues [132] in 22 patients with advanced solid tumors. Furthermore, infusion-related hypersensitivity reactions, probably caused by the liposomal formulation, were observed with all dosages administered (1, 2, 4, 6 mg/kg per week). Phase II trials are currently underway and results are pending. But can the discrepancy between positive responses in *in vitro* studies and their lack in clinical trials be explained? First of all, this targeted therapy was completely untargeted in all of these phase II trials. For example, C-RAF AON was administered to patients diagnosed with prostate or colon carcinomas – nevertheless, no single study describing C-RAF overexpression in these tumor entities has yet been published. Furthermore, no single patient enrolled in these phase II studies was shown to exhibit C-RAF overexpression in tumor samples prior to drug administration. A recent study from Mullen and colleagues [134] further underscores the importance of patient preselection. They observed a correlation between growth inhibition by ISIS 5132 *in vitro* and a high contribution of C-RAF to total (A-, B- and C-RAF) expression levels. Next, many targeted therapies (with the exception of imatinib mesylate in the treatment of BCR-ABL-positive chronic myeloid leukemia) disappointed in clinical trials, when administered as single agents. However, as part of combination therapies or even as part of polychemotherapies, they demonstrated their real clinical benefit. In support of these observations, superadditive effects of ISIS 5132 and LErafAON could be demonstrated in cell lines when coadministered with common cytotoxic agents [135–137].

Considering the frequent mutational activation of B-RAF described in human tumors [5], it remains doubt-

Table 1. Summary of clinical studies conducted with the RAF AONs ISIS 5132 and LErafAON, the RAF kinase inhibitor BAY 43-9006 and the MEK inhibitor CI-1040.

Drug	Study	Combination	Phase	Patients eligible for response	PD	SD	PR	CR
ISIS 5132	Cunningham et al. [126]	single agent	I	34	27	6	1	0
	Stevenson et al. [124]	single agent	I	29	27	2	0	0
	Rudin et al. [125]	single agent	I	13	8	5	0	0
	Oza et al. [131]	single agent	II	16	12	4	0	0
	Tolcher et al. [130]	single agent	II	16	15	1	0	0
	Cripps et al. [129]	single agent	II	15	10	5	0	0
	Coudert et al. [128]	single agent	II	10	10	0	0	0
LErafAON	Rudin et al. [132]	single agent	I	14	9	5	0	0
BAY 43-9006	Awada et al. [144]	single agent	I	32	15	16	1	0
	Clark et al. [142]	single agent	I	17	12	5	0	0
	Strumberg et al. [145]	single agent	I	45	19	25	1	0
	Moore et al. [143]	single agent	I	41	32	9	0	0
	Kupsch et al. [146]	BAY 43-9006 + oxaliplatin	I	32	13	17	2	0
	ASCO 2005 abstr. 3062	single agent	I	31	27	2	2	0
	ASCO 2004 abstr. 6611	single agent	I	27	23		4	0
	ASCO 2005 abstr. 3037	single agent	I/II	22	9	12	1	0
	ASCO 2004 abstr. 4501	single agent	II	63	15	18	25	0
	ASCO 2005 abstr. 4762	single agent	II	4	3	1	0	0
	ASCO 2005 abstr. 5566	single agent	II	10	4	6	0	0
	ASCO 2004 abstr. 7506	single agent	II	19	15	3	1	0
	ASCO 2005 abstr. 4510	single agent	III	24 vs 12 weeks PFS ($p < 0.00001$)				
	ASCO 2004 abstr. 3049	BAY 43-9006 + doxorubicin	I	34	17	16	1	0
	ASCO 2005 abstr. 3115	BAY 43-9006 + irinotecan	I	27	10	17	0	0
	ASCO 2005 abstr. 7508	BAY 43-9006 + dacarbazine	I	11	6	3	2	0
	ASCO 2005 abstr. 3067	BAY 43-9006 + gefitinib	I	12	3	8	1	0
	ASCO 2004 abstr. 3059	BAY 43-9006 + gemcitabine	I/II	42	16	23	3	0
	ASCO 2004 abstr. 7507	BAY 43-9006 + carboplatin + paclitaxel	I/II	32	2	19	11	0
CI-1040	Lorusso et al. [152]	single agent	I	41	21	19	1	0
	Rinehart et al. [153]	single agent	II	52	44	8	0	0

Wherever possible, the evaluation of the clinical response has been reviewed. PD, progressive disease; SD, stable disease; PR, partial response; CR, complete response; PFS, progression-free survival.

ful if targeting C-RAF mRNA rather than the other isoforms will remain a viable strategy. In one recent study with ovarian cancer cells, which also used AONs against A-RAF (ISIS 15489) and B-RAF (ISIS 15344), McPhillips and colleagues [138] could show that C-RAF was most relevant for carcinogenesis and therefore has to be considered the primary target for antisense approaches. This may not be too surprising given the fact that C-RAF is involved in the signal transduction from deregulated RAS and RTKs, but also some forms of mutant B-RAF, which require C-RAF for MEK activation (Fig. 1). Targeting the appropriate RAF protein in a given tumor will require information on the expression as well as the mutational and functional status of all isoforms.

RAF kinase inhibitors

Several RAF kinase inhibitors such as ZM336372 (AstraZeneca, Macclesfield, UK), L-779450 (Merck, Darmstadt, Germany), or oxindole derivates (GlaxoSmithKline, Brentford, UK) exist, but the most established and tested is BAY 43-9006 (Sorafenib, Bayer AG, Leverkusen, Germany/Onyx Pharmaceuticals, Richmond, Calif.). This bi-aryl urea was designed as a small-molecule inhibitor of C-RAF and B-RAF [139] but additional characterization revealed a much broader range of targets. Among many others it efficiently blocked FLT3, c-KIT and pro-angiogenic receptor tyrosine kinases such as VEGFR-2, VEGFR-3 and mPDGFR-β [140]. *In vitro* studies demonstrated inhibition of the RAF-MEK-ERK pathway in several tumor cell lines. Subsequent *in vivo* studies proved

a broad-spectrum antitumor activity in several human tumor xenograft models [140, 141]. Four phase I trials, a German, a Canadian, a Belgian and an American have been published [142–145] and many more are currently ongoing – some of them already presented as abstracts (www.asco.org). Beside a satisfying rate of disease stabilizations, a few tumor regressions have also been noticed (Table 1). In contrast to RAF AONs, clinicians have already started testing BAY 43-9006 as part of combination therapies. A recently published phase I trial, combining BAY 43-9006 with oxaliplatin, revealed a benefit in patients with refractory solid tumors [146]. Further studies are currently ongoing, and again, promising preliminary results have already been presented as abstracts (Table 1). In addition to phase I combination trials, several phase II trials administering BAY 43-9006 as a single agent or as part of combination therapies are currently ongoing (Table 1). Preliminary results of a phase II randomized discontinuation trial were particularly interesting in the subgroup of patients with kidney cancer (Ratain et al., ASCO 2004, abstract 4501). Approximately 40% of patients responded (> 25% tumor reduction) and thus were continued with BAY 43-9006, and another 30% of treated patients showed stable disease (defined as a response between 25% tumor reduction and 25% tumor growth). These patients were randomized to receive 400 mg bid oral placebo or BAY 43-9006. The therapeutic outcome was significantly superior in the BAY 43-9006 group (ASCO 2005, abstract 4544). Recently, this drug entered the stage of clinical phase III trials. One randomized trial comparing BAY 43-9006 with placebo and best supportive care in patients with kidney cancer has already been presented in abstract form and revealed a significantly prolonged progression-free survival for patients treated with BAY 43-9006 (Table 1) (Escudier et al, ASCO 2005, abstract. 4510). Despite these promising results in kidney cancer, it remains unclear if VEGF rather than RAF is the relevant target of BAY 43-9006, as VEGF overexpression can be observed in the majority of cases [147]. Furthermore, clinical trials with other drugs targeted at VEGF, such as the monoclonal anti-VEGF antibody bevacizumab (rhuMab VEGF, Avastin, Gentech, South San Francisco, Calif.) or the small-molecule VEGFR inhibitor SU11248 (Sutent Pfizer, La Jolla, Calif.), were equally successful [148].

New methods targeting RAF

Beside these two established therapeutic approaches, new methods targeting RAF are being developed, some of them showing promising results in preclinical studies. For example, Gentschev and colleagues [149] demonstrated a significantly reduced tumor growth in two transgenic mouse models of RAF-oncogene-induced lung adenomas through a new live C-RAF vaccine based on an attenuated *Salmonella enterica* serovar Typhimurium *aroA* strain [149].

Targeting MEK

Targeting MEK provides an additional means to interfere with RAS-RAF-MEK-ERK signaling. Although various MEK-ERK-independent functions have been discussed, MEK is certainly the main effector of the RAF kinases. Again, several MEK inhibitors exist (PD98059; Pfizer; UO126; DuPont Pharmaceuticals) but due to its oral availability, CI-1040 (PD-0184352; Pfizer) was the first to be tested in clinical trials. In a preclinical study performed by Kramer and colleagues [150], its efficacy was compared with BAY 43-9006 in RAF-dependent lung tumor mouse models. Both drugs were equally effective in abrogating RAF-MEK-ERK signal transduction as assessed by ERK phosphorylation. CI-1040 further reduced adenoma formation to a third and significantly restored lung structure, while BAY 43-9006 did not. The authors try to explain this interesting finding through differences in the *in vivo* accessibility of these inhibitors to subcellular sites where C-RAF is localized. As another possible explanation, they took the different regulation of RAF and MEK. RAF is regulated by a complex system of activatory and inhibitory events which makes it difficult to potently inhibit full RAF activity. Hall-Jackson and colleagues [151] further observed a paradoxical activation of RAF when they tried to inhibit it by ZM 336372, which they explained through the existence of feedback loops. The regulation of MEK is less complex and easier to achieve in the living organism. Considering these promising results, phase I and II trials administering CI-1040 to patients with advanced cancer started in 2004 [152, 153]. Unfortunately, results were disappointing, with only one single partial response. Currently, clinical development with the second-generation MEK inhibitor PD 0325901, which has markedly superior pharmacologic and biopharmaceutical properties, including a more than 50-fold increased potency against MEK [153], is underway. Another interesting finding concerning the efficacy of MEK inhibitors was recently published by Solit and colleagues [154]. They demonstrated that cell lines harboring the B-RAF V600E mutation are more susceptible to MEK inhibitors than those without mutant B-RAF. Even cell lines with RAS mutations were less affected, presumably because multiple other pathways can be stimulated by activated RAS [155]. They conclude that restricting patient collectives for future studies with MEK inhibitors to those with mutated B-RAF might substantially increase the clinical benefit of these drugs.

In conclusion, targeted anti-RAF therapy, especially with the RAF kinase inhibitor BAY 43-9006, definitely has the potential to provide therapeutic alternatives. There is a rapid progression of these new substance into the clinic.

However, despite the time factor, careful patient profiling before their inclusion in clinical studies will be an inevitable step in order to realize the full therapeutic benefit of these new drugs.

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1 Rapp U. R., Cleveland J. L., Storm S. M., Beck T. W. and Huleihel M. (1986) Transformation by raf and myc oncogenes. Princess Takamatsu Symp. **17:** 55–74
2 Rapp U. R., Goldsborough M. D., Mark G. E., Bonner T. I., Groffen J., Reynolds F. H. Jr and et al. (1983) Structure and biological activity of v-raf, a unique oncogene transduced by a retrovirus. Proc. Natl. Acad. Sci. USA **80:** 4218–4222
3 Rapp U. R., Cleveland J. L., Bonner T. I. and Storm S. M. (1988) The raf oncogenes. In: The Oncogene Handbook, pp. 213–253, Reddy, E. P., Skalka, A. M. and Curran, T. (eds.), Elsevier, Amsterdam
4 Daum G., Eisenmann-Tappe I., Fries H. W., Troppmair J. and Rapp U. R. (1994) The ins and outs of Raf kinases. Trends Biochem. Sci. **19:** 474–480
5 Wellbrock C., Karasarides M. and Marais R. (2004) The RAF proteins take centre stage. Nat. Rev. Mol. Cell Biol. **5:** 875–885
6 Chen Z., Gibson F., Robinson F., Silvestro L., Pearson G., Xu B. et al. (2001) MAP kinases. Chem. Rev. **101:** 2449–2476
7 Avruch J., Khokhlatchev A., Kyriakis J. M., Luo Z., Tzivion G., Vavvas D. et al. (2001) Ras activation of the Raf kinase: tyrosine kinase recruitment of the MAP kinase cascade. Recent Prog. Horm. Res. **56:** 127–155
8 Kolch W. (2000) Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem. J. **351:** 289–305
9 Kerkhoff E. and Rapp U. R. (2001) The Ras-Raf relationship: an unfinished puzzle. Adv. Enzyme Regul. **41:** 261–267
10 Chong H., Vikis H. G. and Guan K. L. (2003) Mechanisms of regulating the Raf kinase family. Cell Signal. **15:** 463–469
11 Dougherty M. K. and Morrison D. K. (2004) Unlocking the code of 14-3-3. J. Cell Sci. **117:** 1875–1884
12 Bos J. L. (1995) p21ras: an oncoprotein functioning in growth factor-induced signal transduction. Eur. J. Cancer **31A:** 1051–1054
13 Hancock J. F. (2003) Ras proteins: different signals from different locations. Nat. Rev. Mol. Cell Biol. **4:** 373–384
14 Luo Z., Tzivion G., Belshaw P. J., Vavvas D., Marshall M. and Avruch J. (1996) Oligomerization activates c-Raf-1 through a Ras-dependent mechanism. Nature **383:** 181–185
15 Farrar M. A., Alberol I. and Perlmutter R. M. (1996) Activation of the Raf-1 kinase cascade by coumermycin-induced dimerization. Nature **383:** 178–181
16 Weber C. K., Slupsky J. R., Kalmes H. A. and Rapp U. R. (2001) Active Ras induces heterodimerization of cRaf and BRaf. Cancer Res. **61:** 3595–3598
17 Wan P. T., Garnett M. J., Roe S. M., Lee S., Niculescu-Duvaz D., Good V. M. et al. (2004) Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell **116:** 855–867
18 Garnett M. J., Rana S., Paterson H., Barford D. and Marais R. (2005) Wild-type and mutant B-RAF activate C-RAF through distinct mechanisms involving heterodimerization. Mol. Cell **20:** 963–969
19 Cowley S., Paterson H., Kemp P. and Marshall C. J. (1994) Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell **77:** 841–852
20 Troppmair J., Bruder J. T., Munoz H., Lloyd P. A., Kyriakis J., Banerjee P. et al. (1994) Mitogen-activated protein kinase/extracellular signal-regulated protein kinase activation by oncogenes, serum, and 12-O-tetradecanoylphorbol-13-acetate requires Raf and is necessary for transformation. J. Biol. Chem. **269:** 7030–7035
21 Bruder J. T., Heidecker G. and Rapp U. R. (1992) Serum-, TPA-, and Ras-induced expression from Ap-1/Ets-driven promoters requires Raf-1 kinase. Genes Dev. **6:** 545–556
22 Wang H. G., Rapp U. R. and Reed J. C. (1996) Bcl-2 targets the protein kinase Raf-1 to mitochondria. Cell **87:** 629–638
23 Alavi A., Hood J. D., Frausto R., Stupack D. G. and Cheresh D. A. (2003) Role of Raf in vascular protection from distinct apoptotic stimuli. Science **301:** 94–96
24 Jin S., Zhuo Y., Guo W. and Field J. (2005) p21-activated kinase 1 (Pak1)-dependent phosphorylation of Raf-1 regulates its mitochondrial localization, phosphorylation of BAD, and Bcl-2 association. J. Biol. Chem. **280:** 24698–24705
25 Kuznetsov A. V., Janakiraman M., Margreiter R. and Troppmair J. (2004) Regulating cell survival by controlling cellular energy production: novel functions for ancient signaling pathways? FEBS Lett. **577:** 1–4
26 Le Roy C. and Wrana J. L. (2005) Clathrin- and non-clathrin-mediated endocytic regulation of cell signalling. Nat. Rev. Mol. Cell Biol. **6:** 112–126
27 Teis D. and Huber L. A. (2003) The odd couple: signal transduction and endocytosis. Cell Mol. Life Sci. **60:** 2020–2033
28 Plowman S. J. and Hancock J. F. (2005) Ras signaling from plasma membrane and endomembrane microdomains. Biochim. Biophys. Acta. **1746:** 274–283
29 Chiu V. K., Bivona T., Hach A., Sajous J. B., Silletti J., Wiener H. et al. (2002) Ras signalling on the endoplasmic reticulum and the Golgi. Nat. Cell Biol. **4:** 343–350
30 Morrison D. K. and Davis R. J. (2003) Regulation of MAP kinase signaling modules by scaffold proteins in mammals. Annu. Rev. Cell Dev. Biol. **19:** 91–118
31 Yeung K., Seitz T., Li S., Janosch P., McFerran B., Kaiser C. et al. (1999) Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP. Nature **401:** 173–177
32 Odabaei G., Chatterjee D., Jazirehi A. R., Goodglick L., Yeung K. and Bonavida B. (2004) Raf-1 kinase inhibitor protein: structure, function, regulation of cell signaling, and pivotal role in apoptosis. Adv. Cancer Res. **91:** 169–200
33 Trakul N. and Rosner M. R. (2005) Modulation of the MAP kinase signaling cascade by Raf kinase inhibitory protein. Cell Res. **15:** 19–23
34 Peyssonnaux C. and Eychene A. (2001) The Raf/MEK/ERK pathway: new concepts of activation. Biol. Cell **93:** 53–62
35 Raabe T. (2000) The sevenless signaling pathway: variations of a common theme. Biochim. Biophys. Acta **1496:** 151–163
36 Freeman M. (1997) Cell determination strategies in the *Drosophila* eye. Development **124:** 261–270
37 Kurada P. and White K. (1999) Epidermal growth factor receptor: its role in *Drosophila* eye differentiation and cell survival. Apoptosis **4:** 239–243
38 Sternberg P. W. and Han M. (1998) Genetics of RAS signaling in *C. elegans*. Trends Genet. **14:** 466–472
39 Wurgler-Murphy S. M. and Saito H. (1997) Two-component signal transducers and MAPK cascades. Trends Biochem. Sci. **22:** 172–176
40 The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature **408:** 796–815
41 Bleecker A. B. and Kende H. (2000) Ethylene: a gaseous signal molecule in plants. Annu. Rev. Cell Dev. Biol. **16:** 1–18
42 Johnson P. R. and Ecker J. R. (1998) The ethylene gas signal transduction pathway: a molecular perspective. Annu. Rev. Genet. **32:** 227–254

43 Davies H., Bignell G. R., Cox C., Stephens P., Edkins S., Clegg S. et al. (2002) Mutations of the BRAF gene in human cancer. Nature **417:** 949–954

44 Zebisch A., Staber P. B., Delavar A., Bodner C., Hiden K., Fischereder K. et al. (2006) Two transforming C-RAF germline mutations identified in patients with therapy-related acute myeloid leukemia. Cancer Res. **66:** 3401-3408

45 Robinson M. J., Stippec S. A., Goldsmith E., White M. A. and Cobb M. H. (1998) A constitutively active and nuclear form of the MAP kinase ERK2 is sufficient for neurite outgrowth and cell transformation. Curr. Biol. **8:** 1141–1150

46 Baumann B., Weber C. K., Troppmair J., Whiteside S., Israel A., Rapp U. R. et al. (2000) Raf induces NF-kappaB by membrane shuttle kinase MEKK1, a signaling pathway critical for transformation. Proc. Natl. Acad. Sci. USA **97:** 4615–4620

47 Schulze A., Lehmann K., Jefferies H. B., McMahon M. and Downward J. (2001) Analysis of the transcriptional program induced by Raf in epithelial cells. Genes Dev. **15:** 981–994

48 Gise A. von, Lorenz P., Wellbrock C., Hemmings B., Berberich-Siebelt F., Rapp U. R. et al. (2001) Apoptosis suppression by Raf-1 and MEK1 requires MEK- and phosphatidylinositol 3-kinase-dependent signals. Mol. Cell Biol. **21:** 2324–2336

49 Rennefahrt U., Janakiraman M., Ollinger R. and Troppmair J. (2005) Stress kinase signaling in cancer: fact or fiction? Cancer Lett. **217:** 1–9

50 Boland C. R. and Ricciardiello L. (1999) How many mutations does it take to make a tumor? Proc. Natl. Acad. Sci. USA **96:** 14675–14677

51 Sherr C. J. (2000) The Pezcoller lecture: cancer cell cycles revisited. Cancer Res. **60:** 3689–3695

52 Kerkhoff E. and Rapp U. R. (1998) High-intensity Raf signals convert mitotic cell cycling into cellular growth. Cancer Res. **58:** 1636–1640

53 Sewing A., Wiseman B., Lloyd A. C. and Land H. (1997) High-intensity Raf signal causes cell cycle arrest mediated by p21Cip1. Mol. Cell Biol. **17:** 5588–5597

54 Kerkhoff E. and Rapp U. R. (1998) Cell cycle targets of Ras/Raf signalling. Oncogene **17:** 1457–1462

55 Kroemer G. and Reed J. C. (2000) Mitochondrial control of cell death. Nat. Med. **6:** 513–519

56 Troppmair J. and Rapp U. R. (2003) Raf and the road to cell survival: a tale of bad spells, ring bearers and detours. Biochem. Pharmacol. **66:** 1341–1345

57 Brazil D. P. and Hemmings B. A. (2001) Ten years of protein kinase B signalling: a hard Akt to follow. Trends. Biochem. Sci. **26:** 657–664

58 Reed J. C. (1998) Bcl-2 family proteins. Oncogene **17:** 3225–3236

59 Vaux D. L. and Silke J. (2005) IAPs, RINGs and ubiquitylation. Nat. Rev. Mol. Cell Biol. **6:** 287–297

60 Cleveland J. L., Troppmair J., Packham G., Askew D. S., Lloyd P., Gonzalez-Garcia M. et al. (1994) v-raf suppresses apoptosis and promotes growth of interleukin-3-dependent myeloid cells. Oncogene **9:** 2217–2226

61 Huser M., Luckett J., Chiloeches A., Mercer K., Iwobi M., Giblett S. et al. (2001) MEK kinase activity is not necessary for Raf-1 function. EMBO J. **20:** 1940–1951

62 Mikula M., Schreiber M., Husak Z., Kucerova L., Ruth J., Wieser R. et al. (2001) Embryonic lethality and fetal liver apoptosis in mice lacking the c-raf-1 gene. EMBO J. **20:** 1952–1962

63 Zhong J., Troppmair J. and Rapp U. R. (2001) Independent control of cell survival by Raf-1 and Bcl-2 at the mitochondria. Oncogene **20:** 4807–4816

64 Erhardt P., Schremser E. J. and Cooper G. M. (1999) B-Raf inhibits programmed cell death downstream of cytochrome c release from mitochondria by activating the MEK/Erk pathway. Mol. Cell Biol. **19:** 5308–5315

65 Wiese S., Pei G., Karch C., Troppmair J., Holtmann B., Rapp U. R. et al. (2001) Specific function of B-Raf in mediating survival of embryonic motoneurons and sensory neurons. Nat. Neurosci. **4:** 137–142

66 Chittenden T. (2002) BH3 domains: intracellular death-ligands critical for initiating apoptosis. Cancer Cell **2:** 165–166

67 O'Neill E., Rushworth L., Baccarini M. and Kolch W. (2004) Role of the kinase MST2 in suppression of apoptosis by the proto-oncogene product Raf-1. Science **306:** 2267–2270

68 Chen J., Fujii K., Zhang L., Roberts T. and Fu H. (2001) Raf-1 promotes cell survival by antagonizing apoptosis signal-regulating kinase 1 through a MEK-ERK independent mechanism. Proc. Natl. Acad. Sci. USA **98:** 7783–7788

69 Fabian J. R., Daar I. O. and Morrison D. K. (1993) Critical tyrosine residues regulate the enzymatic and biological activity of Raf-1 kinase. Mol. Cell Biol. **13:** 7170–7179

70 Bosch E., Cherwinski H., Peterson D. and McMahon M. (1997) Mutations of critical amino acids affect the biological and biochemical properties of oncogenic A-Raf and Raf-1. Oncogene **15:** 1021–1033

71 Woods D., Parry D., Cherwinski H., Bosch E., Lees E. and McMahon M. (1997) Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by p21Cip. Mol. Cell Biol. **17:** 5598–5611

72 O'Neill E. E., Matallanas D. and Kolch W. (2005) Mammalian sterile 20-like kinases in tumor suppression: an emerging pathway. Cancer Res. **65:** 5485–5487

73 Weston C. R., Balmanno K., Chalmers C., Hadfield K., Molton S. A., Ley R. et al. (2003) Activation of ERK1/2 by deltaRaf-1: ER* represses Bim expression independently of the JNK or PI3K pathways. Oncogene **22:** 1281–1293

74 Plas D. R. and Thompson C. B. (2002) Cell metabolism in the regulation of programmed cell death. Trends Endocrinol. Metab. **13:** 75–78

75 Le Mellay V., Houben R., Troppmair J., Hagemann C., Mazurek S., Frey U. et al. (2002) Regulation of glycolysis by Raf protein serine/threonine kinases. Adv. Enzyme Regul. **42:** 317–332

76 Rajalingam K., Wunder C., Brinkmann V., Churin Y., Hekman M., Sievers C. et al. (2005) Prohibitin is required for Ras-induced Raf-MEK-ERK activation and epithelial cell migration. Nat. Cell Biol. **7:** 837–843

77 Nijtmans L. G., Artal S. M., Grivell L. A. and Coates P. J. (2002) The mitochondrial PHB complex: roles in mitochondrial respiratory complex assembly, ageing and degenerative disease. Cell Mol. Life Sci. **59:** 143–155

78 Evers E. E., Zondag G. C., Malliri A., Price L. S., Klooster J. P. ten, Kammen R. A. van der et al. (2000) Rho family proteins in cell adhesion and cell migration. Eur. J. Cancer **36:** 1269–1274

79 Watanabe N., Kato T., Fujita A., Ishizaki T. and Narumiya S. (1999) Cooperation between mDia1 and ROCK in Rho-induced actin reorganization. Nat. Cell Biol. **1:** 136–143

80 Ehrenreiter K., Piazzolla D., Velamoor V., Sobczak I., Small J. V., Takeda J. et al. (2005) Raf-1 regulates Rho signaling and cell migration. J. Cell Biol. **168:** 955–964

81 Pritchard C. A., Hayes L., Wojnowski L., Zimmer A., Marais R. M. and Norman J. C. (2004) B-Raf acts via the ROCKII/LIMK/cofilin pathway to maintain actin stress fibers in fibroblasts. Mol. Cell Biol. **24:** 5937–5952

82 Wojnowski L., Stancato L. F., Zimmer A. M., Hahn H., Beck T. W., Larner A. C. et al. (1998) Craf-1 protein kinase is essential for mouse development. Mech. Dev. **76:** 141–149

83 Wojnowski L., Zimmer A. M., Beck T. W., Hahn H., Bernal R., Rapp U. R. et al. (1997) Endothelial apoptosis in Braf-deficient mice. Nat. Genet. **16:** 293–297

84 Ikenoue T., Hikiba Y., Kanai F., Aragaki J., Tanaka Y., Imamura J. et al. (2004) Different effects of point mutations within the B-Raf glycine-rich loop in colorectal tumors on mitogen-activated protein/extracellular signal-regulated kinase kinase/

extracellular signal-regulated kinase and nuclear factor kappaB pathway and cellular transformation. Cancer Res. **64:** 3428–3435
85 Ikenoue T., Kanai F., Hikiba Y., Tanaka Y., Imamura J., Ohta M. et al. (2005) Functional consequences of mutations in a putative Akt phosphorylation motif of B-raf in human cancers. Mol. Carcinog. **43:** 59–63
86 Wellbrock C., Ogilvie L., Hedley D., Karasarides M., Martin J., Niculescu-Duvaz D. et al. (2004) V599EB-RAF is an oncogene in melanocytes. Cancer Res. **64:** 2338–2342
87 Knauf J. A., Ma X., Smith E. P., Zhang L., Mitsutake N., Liao X. H. et al. (2005) Targeted expression of BRAFV600E in thyroid cells of transgenic mice results in papillary thyroid cancers that undergo dedifferentiation. Cancer Res. **65:** 4238–4245
88 Hingorani S. R., Jacobetz M. A., Robertson G. P., Herlyn M. and Tuveson D. A. (2003) Suppression of BRAF(V599E) in human melanoma abrogates transformation. Cancer Res. **63:** 5198–5202
89 Karasarides M., Chiloeches A., Hayward R., Niculescu-Duvaz D., Scanlon I., Friedlos F. et al. (2004) B-RAF is a therapeutic target in melanoma. Oncogene **23:** 6292–6298
90 Sharma A., Trivedi N. R., Zimmerman M. A., Tuveson D. A., Smith C. D. and Robertson G. P. (2005) Mutant V599EB-Raf regulates growth and vascular development of malignant melanoma tumors. Cancer Res. **65:** 2412–2421
91 Mitsutake N., Knauf J. A., Mitsutake S., Mesa C. Jr, Zhang L. and Fagin J. A. (2005) Conditional BRAFV600E expression induces DNA synthesis, apoptosis, dedifferentiation, and chromosomal instability in thyroid PCCL3 cells. Cancer Res. **65:** 2465–2473
92 Michaloglou C., Vredeveld L. C., Soengas M. S., Denoyelle C., Kuilman T., Horst C. M. van der et al. (2005) BRAFE600-associated senescence-like cell cycle arrest of human naevi. Nature **436:** 720–724
93 Rajagopalan H., Bardelli A., Lengauer C., Kinzler K. W., Vogelstein B. and Velculescu V. E. (2002) Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. Nature **418:** 934
94 Chan T. L., Zhao W., Leung S. Y. and Yuen S. T. (2003) BRAF and KRAS mutations in colorectal hyperplastic polyps and serrated adenomas. Cancer Res. **63:** 4878–4881
95 Yuen S. T., Davies H., Chan T. L., Ho J. W., Bignell G. R., Cox C. et al. (2002) Similarity of the phenotypic patterns associated with BRAF and KRAS mutations in colorectal neoplasia. Cancer Res. **62:** 6451–6455
96 Lee S. H., Lee J. W., Soung Y. H., Kim H. S., Park W. S., Kim S. Y. et al. (2003) BRAF and KRAS mutations in stomach cancer. Oncogene **22:** 6942–6945
97 Ishimura N., Yamasawa K., Karim Rumi M. A., Kadowaki Y., Ishihara S., Amano Y. et al. (2003) BRAF and K-ras gene mutations in human pancreatic cancers. Cancer Lett. **199:** 169–173
98 Kimura E. T., Nikiforova M. N., Zhu Z., Knauf J. A., Nikiforov Y. E. and Fagin J. A. (2003) High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. Cancer Res. **63:** 1454–1457
99 Soares P., Trovisco V., Rocha A. S., Lima J., Castro P., Preto A. et al. (2003) BRAF mutations and RET/PTC rearrangements are alternative events in the etiopathogenesis of PTC. Oncogene **22:** 4578–4580
100 Xu X., Quiros R. M., Gattuso P., Ain K. B. and Prinz R. A. (2003) High prevalence of BRAF gene mutation in papillary thyroid carcinomas and thyroid tumor cell lines Cancer Res. **63:** 4561–4567
101 Xing M. (2005) BRAF mutation in thyroid cancer. Endocr. Relat. Cancer **12:** 245–262
102 Daniotti M., Oggionni M., Ranzani T., Vallacchi V., Campi V., Di Stasi D. et al. (2004) BRAF alterations are associated with complex mutational profiles in malignant melanoma. Oncogene **23:** 5968–5977
103 Patton E. E., Widlund H. R., Kutok J. L., Kopani K. R., Amatruda J. F., Murphey R. D. et al. (2005) BRAF mutations are sufficient to promote nevi formation and cooperate with p53 in the genesis of melanoma. Curr. Biol. **15:** 249–254
104 Tsao H., Goel V., Wu H., Yang G. and Haluska F. G. (2004) Genetic interaction between NRAS and BRAF mutations and PTEN/MMAC1 inactivation in melanoma. J. Invest. Dermatol. **122:** 337–341
105 Porra V., Ferraro-Peyret C., Durand C., Selmi-Ruby S., Giroud H., Berger-Dutrieux N. et al. (2005) Silencing of the tumor suppressor gene SLC5A8 is associated with BRAF mutations in classical papillary thyroid carcinomas. J. Clin. Endocrinol. Metab. **90:** 3028–3035
106 Olipitz W., Hopfinger G., Aguiar R. C., Gunsilius E., Girschikofsky M., Bodner C. et al. (2002) Defective DNA-mismatch repair: a potential mediator of leukemogenic susceptibility in therapy-related myelodysplasia and leukemia. Genes Chromosomes Cancer **34:** 243–248
107 Yee C. J., Roodi N., Verrier C. S. and Parl F. F. (1994) Microsatellite instability and loss of heterozygosity in breast cancer. Cancer Res. **54:** 1641–1644
108 Wang L., Cunningham J. M., Winters J. L., Guenther J. C., French A. J., Boardman L. A. et al. (2003) BRAF mutations in colon cancer are not likely attributable to defective DNA mismatch repair. Cancer Res. **63:** 5209–5212
109 Storm S. M. and Rapp U. R. (1993) Oncogene activation: c-raf-1 gene mutations in experimental and naturally occurring tumors. Toxicol. Lett. **67:** 201–210
110 Emuss V., Garnett M., Mason C. and Marais R. (2005) Mutations of C-RAF are rare in human cancer because C-RAF has a low basal kinase activity compared with B-RAF. Cancer Res. **65:** 9719–9726
111 Sill H., Olipitz W. and Schimek M. G. (2005) Therapy-related myelodysplastic syndrome and acute myeloid leukemia after autologous bone marrow transplantation: dosis facit venenum? J. Clin. Oncol. **23:** 8120–8121
112 Kerkhoff E., Fedorov L. M., Siefken R., Walter A. O., Papadopoulos T. and Rapp U. R. (2000) Lung-targeted expression of the c-Raf-1 kinase in transgenic mice exposes a novel oncogenic character of the wild-type protein. Cell Growth Differ. **11:** 185–190
113 Schmidt C. A., Oettle H., Ludwig W. D., Serke S., Pawlaczyk-Peter B., Wilborn F. et al. (1994) Overexpression of the Raf-1 proto-oncogene in human myeloid leukemia. Leuk. Res. **18:** 409–413
114 Riva C., Lavieille J. P., Reyt E., Brambilla E., Lunardi J. and Brambilla C. (1995) Differential c-myc, c-jun, c-raf and p53 expression in squamous cell carcinoma of the head and neck: implication in drug and radioresistance. Eur. J. Cancer B Oral Oncol. **31B:** 384–391
115 McPhillips F., Mullen P., Monia B. P., Ritchie A. A., Dorr F. A., Smyth J. F. et al. (2001) Association of c-Raf expression with survival and its targeting with antisense oligonucleotides in ovarian cancer. Br. J. Cancer **85:** 1753–1758
116 Ciampi R., Knauf J. A., Kerler R., Gandhi M., Zhu Z., Nikiforova M. N. et al. (2005) Oncogenic AKAP9-BRAF fusion is a novel mechanism of MAPK pathway activation in thyroid cancer. J. Clin. Invest. **115:** 94–101
117 Maldonado J. L., Fridlyand J., Patel H., Jain A. N., Busam K., Kageshita T. et al. (2003) Determinants of BRAF mutations in primary melanomas. J. Natl. Cancer Inst. **95:** 1878–1890
118 Chung G. T., Huang D. P., Lo K. W., Chan M. K. and Wong F. W. (1992) Genetic lesion in the carcinogenesis of cervical cancer. Anticancer Res. **12:** 1485–1490
119 Bernues M., Casadevall C., Miro R., Caballin M. R., Gelabert A., Ejarque M. J. et al. (1998) Analysis of 3p allelic losses in

renal cell carcinomas: comparison with cytogenetic results. Cancer Genet. Cytogenet. **107:** 121–124
120 Ikeda S., Sumii H., Akiyama K., Watanabe S., Ito S., Inoue H. et al. (1989) Amplification of both c-myc and c-raf-1 oncogenes in a human osteosarcoma. Jpn J. Cancer Res. **80:** 6–9
121 Simon R., Richter J., Wagner U., Fijan A., Bruderer J., Schmid U. et al. (2001) High-throughput tissue microarray analysis of 3p25 (RAF1) and 8p12 (FGFR1) copy number alterations in urinary bladder cancer. Cancer Res. **61:** 4514–4519
122 Downward J. (2003) Targeting RAS signalling pathways in cancer therapy. Nat. Rev. Cancer **3:** 11–22
123 Monia B. P., Johnston J. F., Geiger T., Muller M. and Fabbro D. (1996) Antitumor activity of a phosphorothioate antisense oligodeoxynucleotide targeted against C-raf kinase. Nat. Med. **2:** 668–675
124 Stevenson J. P., Yao K. S., Gallagher M., Friedland D., Mitchell E. P., Cassella A. et al. (1999) Phase I clinical/pharmacokinetic and pharmacodynamic trial of the c-raf-1 antisense oligonucleotide ISIS 5132 (CGP 69846A). J. Clin. Oncol. **17:** 2227–2236
125 Rudin C. M., Holmlund J., Fleming G. F., Mani S., Stadler W. M., Schumm P. et al. (2001) Phase I trial of ISIS 5132, an antisense oligonucleotide inhibitor of c-raf-1, administered by 24-hour weekly infusion to patients with advanced cancer. Clin. Cancer Res. **7:** 1214–1220
126 Cunningham C. C., Holmlund J. T., Schiller J. H., Geary R. S., Kwoh T. J., Dorr A. et al. (2000) A phase I trial of c-Raf kinase antisense oligonucleotide ISIS 5132 administered as a continuous intravenous infusion in patients with advanced cancer. Clin. Cancer Res. **6:** 1626–1631
127 O'Dwyer P. J., Stevenson J. P., Gallagher M., Cassella A., Vasilevskaya I., Monia B. P. et al. (1999) c-raf-1 depletion and tumor responses in patients treated with the c-raf-1 antisense oligodeoxynucleotide ISIS 5132 (CGP 69846A). Clin. Cancer Res. **5:** 3977–3982
128 Coudert B., Anthoney A., Fiedler W., Droz J. P., Dieras V., Borner M. et al. (2001) Phase II trial with ISIS 5132 in patients with small-cell (SCLC) and non-small cell (NSCLC) lung cancer: a European Organization for Research and Treatment of Cancer (EORTC) Early Clinical Studies Group report. Eur. J. Cancer **37:** 2194–2198
129 Cripps M. C., Figueredo A. T., Oza A. M., Taylor M. J., Fields A. L., Holmlund J. T. et al. (2002) Phase II randomized study of ISIS 3521 and ISIS 5132 in patients with locally advanced or metastatic colorectal cancer: a National Cancer Institute of Canada clinical trials group study. Clin. Cancer Res. **8:** 2188–2192
130 Tolcher A. W., Reyno L., Venner P. M., Ernst S. D., Moore M., Geary R. S. et al. (2002) A randomized phase II and pharmacokinetic study of the antisense oligonucleotides ISIS 3521 and ISIS 5132 in patients with hormone-refractory prostate cancer. Clin. Cancer Res. **8:** 2530–2535
131 Oza A. M., Elit L., Swenerton K., Faught W., Ghatage P., Carey M. et al. (2003) Phase II study of CGP 69846A (ISIS 5132) in recurrent epithelial ovarian cancer: an NCIC clinical trials group study (NCIC IND.116). Gynecol. Oncol. **89:** 129–133
132 Rudin C. M., Marshall J. L., Huang C. H., Kindler H. L., Zhang C., Kumar D. et al. (2004) Delivery of a liposomal c-raf-1 antisense oligonucleotide by weekly bolus dosing in patients with advanced solid tumors: a phase I study. Clin. Cancer Res. **10:** 7244–7251
133 Gokhale P. C., Zhang C., Newsome J. T., Pei J., Ahmad I., Rahman A. et al. (2002) Pharmacokinetics, toxicity, and efficacy of ends-modified raf antisense oligodeoxyribonucleotide encapsulated in a novel cationic liposome. Clin. Cancer Res. **8:** 3611–3621
134 Mullen P., McPhillips F., MacLeod K., Monia B., Smyth J. F. and Langdon S. P. (2004) Antisense oligonucleotide targeting of Raf-1: importance of raf-1 mRNA expression levels and raf-1-dependent signaling in determining growth response in ovarian cancer. Clin. Cancer Res. **10:** 2100–2108
135 Mewani R. R., Tang W., Rahman A., Dritschilo A., Ahmad I., Kasid U. N. et al. (2004) Enhanced therapeutic effects of doxorubicin and paclitaxel in combination with liposome-entrapped ends-modified raf antisense oligonucleotide against human prostate, lung and breast tumor models. Int. J. Oncol. **24:** 1181–1188
136 Pei J., Zhang C., Gokhale P. C., Rahman A., Dritschilo A., Ahmad I. et al. (2004) Combination with liposome-entrapped, ends-modified raf antisense oligonucleotide (LErafAON) improves the anti-tumor efficacies of cisplatin, epirubicin, mitoxantrone, docetaxel and gemcitabine. Anticancer Drugs **15:** 243–253
137 Geiger T., Muller M., Monia B. P. and Fabbro D. (1997) Antitumor activity of a C-raf antisense oligonucleotide in combination with standard chemotherapeutic agents against various human tumors transplanted subcutaneously into nude mice. Clin. Cancer Res. **3:** 1179–1185
138 McPhillips F., Mullen P., Macleod K. G., Sewell J. M., Monia B. P., Cameron D. A. et al. (2005) Raf-1 is the predominant Raf isoform that mediates growth factor-stimulated growth in ovarian cancer cells. Carcinogenesis **27:** 729–739
139 Lyons J. F., Wilhelm S., Hibner B. and Bollag G. (2001) Discovery of a novel Raf kinase inhibitor. Endocr. Relat. Cancer **8:** 219–225
140 Wilhelm S. M., Carter C., Tang L., Wilkie D., McNabola A., Rong H. et al. (2004) BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res. **64:** 7099–7109
141 Wilhelm S. and Chien D. S. (2002) BAY 43-9006: preclinical data. Curr. Pharm. Des. **8:** 2255–2257
142 Clark J. W., Eder J. P., Ryan D., Lathia C. and Lenz H. J. (2005) Safety and pharmacokinetics of the dual action raf kinase and vascular endothelial growth factor receptor inhibitor, BAY 43-9006, in patients with advanced, refractory solid tumors. Clin. Cancer Res. **11:** 5472–5480
143 Moore M., Hirte H. W., Siu L., Oza A., Hotte S. J., Petrenciuc O. et al. (2005) Phase I study to determine the safety and pharmacokinetics of the novel Raf kinase and VEGFR inhibitor BAY 43-9006, administered for 28 days on/7 days off in patients with advanced, refractory solid tumors. Ann. Oncol. **16:** 1688–1694
144 Awada A., Hendlisz A., Gil T., Bartholomeus S., Mano M., Valeriola D. de et al. (2005) Phase I safety and pharmacokinetics of BAY 43-9006 administered for 21 days on/7 days off in patients with advanced, refractory solid tumours. Br. J. Cancer **92:** 1855–1861
145 Strumberg D., Richly H., Hilger R. A., Schleucher N., Korfee S., Tewes M. et al. (2005) Phase I clinical and pharmacokinetic study of the novel Raf kinase and vascular endothelial growth factor receptor inhibitor BAY 43-9006 in patients with advanced refractory solid tumors. J. Clin. Oncol. **23:** 965–972
146 Kupsch P., Henning B. F., Passarge K., Richly H., Wiesemann K., Hilger R. A. et al. (2005) Results of a phase I trial of sorafenib (BAY 43-9006) in combination with oxaliplatin in patients with refractory solid tumors, including colorectal cancer. Clin. Colorectal Cancer **5:** 188–196
147 Rini B. I. and Small E. J. (2005) Biology and clinical development of vascular endothelial growth factor-targeted therapy in renal cell carcinoma. J. Clin. Oncol. **23:** 1028–1043
148 Rini B. I., Sosman J. A. and Motzer R. J. (2005) Therapy targeted at vascular endothelial growth factor in metastatic renal cell carcinoma: biology, clinical results and future development. BJU Int. **96:** 286–290

149 Gentschev I., Fensterle J., Schmidt A., Potapenko T., Troppmair J., Goebel W. et al. (2005) Use of a recombinant *Salmonella enterica* serovar *Typhimurium* strain expressing C-Raf for protection against C-Raf induced lung adenoma in mice. BMC Cancer **5:** 15
150 Kramer B. W., Gotz R. and Rapp U. R. (2004) Use of mitogenic cascade blockers for treatment of C-Raf induced lung adenoma *in vivo:* CI-1040 strongly reduces growth and improves lung structure. BMC Cancer **4:** 24
151 Hall-Jackson C. A., Eyers P. A., Cohen P., Goedert M., Boyle F. T., Hewitt N. et al. (1999) Paradoxical activation of Raf by a novel Raf inhibitor. Chem. Biol. **6:** 559–568
152 Lorusso P. M., Adjei A. A., Varterasian M., Gadgeel S., Reid J., Mitchell D. Y. et al. (2005) Phase I and pharmacodynamic study of the oral MEK inhibitor CI-1040 in patients with advanced malignancies. J. Clin. Oncol. **23:** 5281–5293
153 Rinehart J., Adjei A. A., Lorusso P. M., Waterhouse D., Hecht J. R., Natale R. B. et al. (2004) Multicenter phase II study of the oral MEK inhibitor, CI-1040, in patients with advanced non-small-cell lung, breast, colon, and pancreatic cancer. J. Clin. Oncol. **22:** 4456–4462
154 Solit D. B., Garraway L. A., Pratilas C. A., Sawai A., Getz G., Basso A. et al. (2006) BRAF mutation predicts sensitivity to MEK inhibition. Nature **439:** 358–362
155 Repasky G. A., Chenette E. J. and Der C. J. (2004) Renewing the conspiracy theory debate: does Raf function alone to mediate Ras oncogenesis? Trends Cell Biol. 14: 639–647