Regulation of RAF protein kinases in ERK signalling

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Abstract | RAF family kinases were among the first oncoproteins to be described more than 30 years ago. They primarily act as signalling relays downstream of RAS, and their close ties to cancer have fuelled a large number of studies. However, we still lack a systems-level understanding of their regulation and mode of action. The recent discovery that the catalytic activity of RAF depends on an allosteric mechanism driven by kinase domain dimerization is providing a vital new piece of information towards a comprehensive model of RAF function. The fact that current RAF inhibitors unexpectedly induce ERK signalling by stimulating RAF dimerization also calls for a deeper structural characterization of this family of kinases.

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The three decades of research that have elapsed since the discovery of RAF proteins have provided a wealth of information pertaining to their molecular function, regulation and biological role in normal and pathological conditions (FIG. 1). Impressively, more than 13,000 publications related to these proteins are listed in PubMed, which attests to their biological importance. Nonetheless, important gaps in our knowledge still preclude an integrated view of the function and regulation of RAF proteins, therefore limiting our capacity to inhibit their uncontrolled activity in disease conditions such as cancer.

In this Review, we first provide a brief account of the early years following the discovery of RAF proteins, which led to their functional connection to the ERK phosphorylation cascade and to the identification of RAF-related pseudokinases, known as kinase suppressor of RAS (KSR) proteins. We then discuss key mechanistic and structural studies that are shaping our current view of RAF and KSR regulation, highlighting prominent unresolved issues. Finally, we present mechanisms underlying oncogenic and pathological RAF signalling and the unforeseen limitations of current RAF inhibitors. Although much less characterized, it should be noted that RAF proteins also control ERK-independent signalling events, such as RHO-mediated cell migration or BCL-2- and Hippo-regulated cell survival. They are not presented here, but the reader is directed to other relevant literature[1].

RAF proteins: the early years

The first decade of RAF research is characterized by the identification of RAF homologues in diverse species as well as by their biochemical and functional characterization. Soon it was recognized that RAF proteins are part of a larger protein network that dictates cell proliferation and differentiation.

The discovery of RAF proteins. The study of cancer-causing retroviruses was in full swing by the end of the 1970s, and the discovery of the *v-raf* (rapidly accelerated fibrosarcoma) oncogene was fuelled by the characterization of a transforming mouse sarcoma virus[2,3]. Soon thereafter, an avian virus was shown to encode a closely related oncogene named *v-mil* (or *v-mht*)[4–6]. Homology to Tyr-selective SRC kinases had been noted at the time, but the v-RAF and v-MIL oncoproteins turned out to be Ser/Thr kinases[7] (FIG. 2a). The first cellular *RAF* proto-oncogene, *CRAF* (also known as *c-RAF* or *RAF1*), was identified from human and rodent cells in 1985 (REFS 8–11). Several studies shortly reported that oncogenic CRAF invariably contained deletions or rearrangements of the non-catalytic amino-terminal region but had an intact kinase domain[12–16]. These observations were consistent with the presence of repressive elements in the N-terminal region, a hypothesis later confirmed with defined mutants[17–20].

Completing the family portrait, two other mammalian homologues, ARAF[21–24] and BRAF[25], were subsequently identified (FIG. 1). As for CRAF, truncation of their N-terminal region unleashed their ability to transform cells[22,25]. Besides their closely related kinase domain, two evolutionarily conserved areas were found in their N-terminal portion[19] (FIG. 2a). The first conserved region (CR1) includes a Cys-rich domain (CRD; also known as C1 domain)[26] and another conserved element that was later recognized as a RAS-binding domain[27] (RBD). The second one (CR2) consists of a stretch rich in Ser and Thr residues.

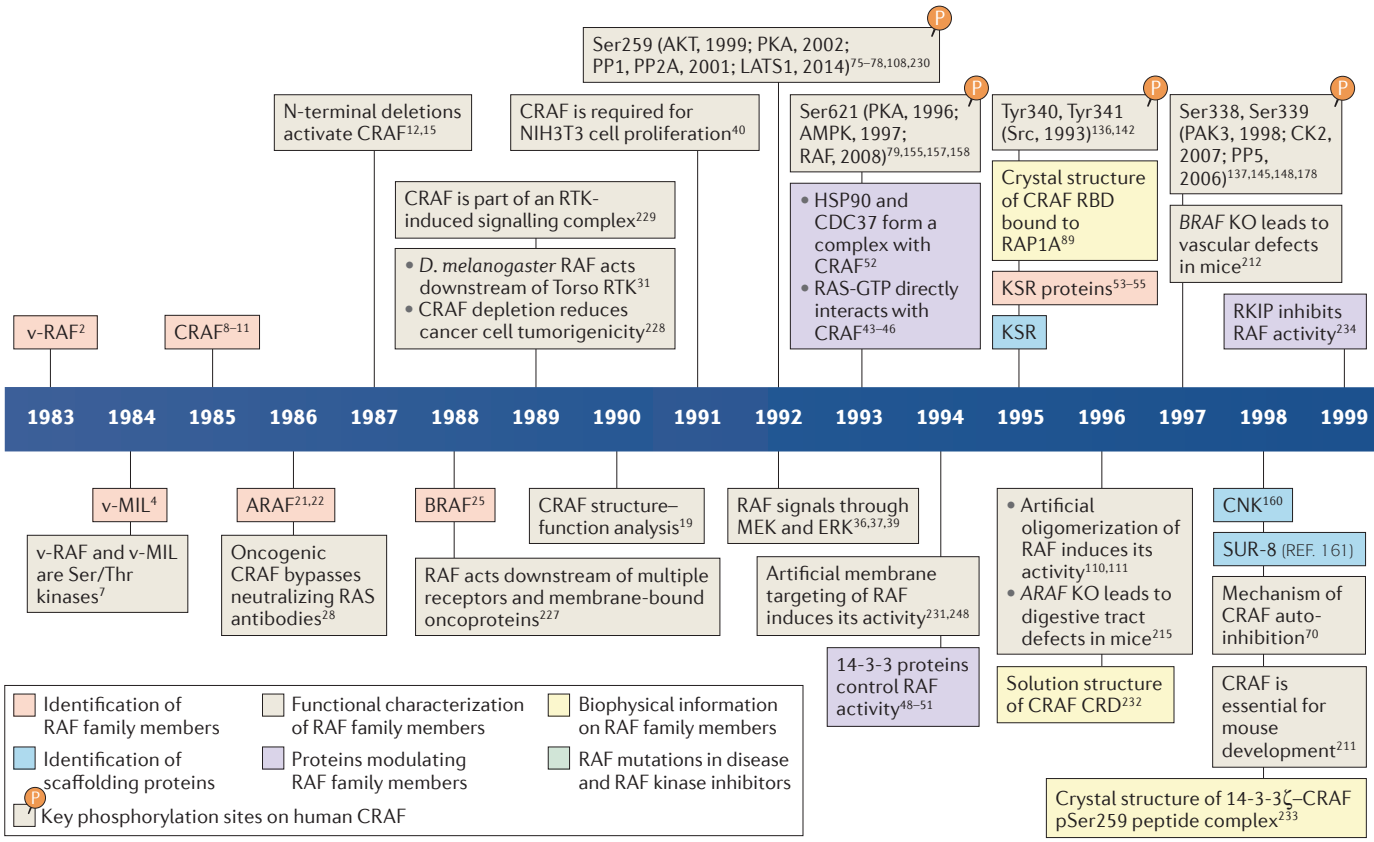
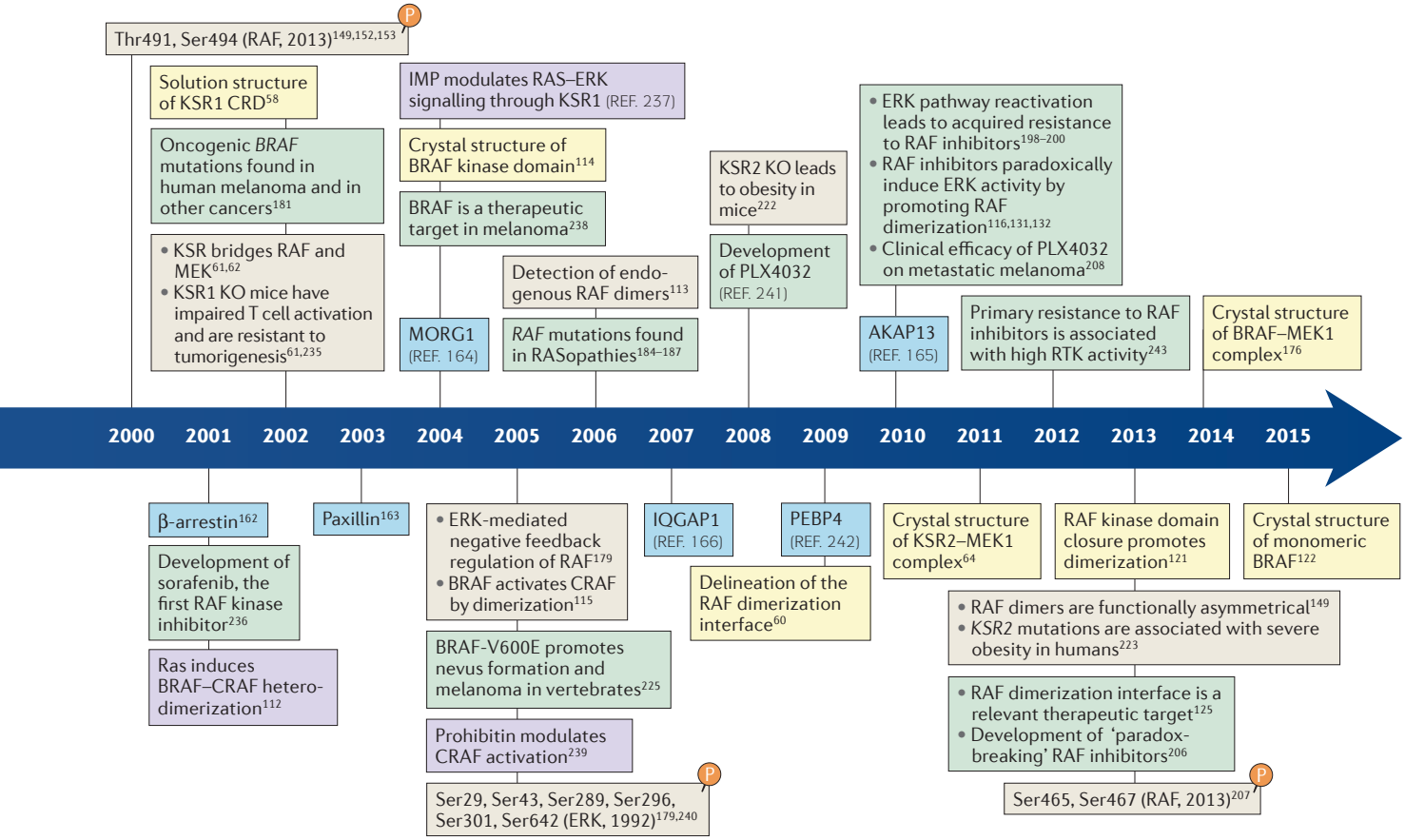


Figure 1 | **Three decades of research on RAF family proteins.** The timeline illustrates significant findings and therapeutic development pertaining to RAF family proteins since their initial discovery. Specific phospho-regulatory sites (orange P) in human CRAF are represented according to the year of their identification. Kinases and phosphatases acting on these sites are indicated in parentheses along with the year of their initial connection to these sites. Note that this is not an exhaustive catalogue of the milestones in the history of RAF research but rather a selection biased by the research interests of the authors. AMPK, AMP-activated protein kinase; AKAP13, A-kinase anchor protein 13; CK2, casein kinase 2; CNK, connector enhancer of KSR; CRD, Cys-rich domain; *D. melanogaster*, *Drosophila melanogaster*; HSP90, heat shock protein 90; IMP, impedes mitogenic signal propagation; IQGAP1, IQ motif containing GTPase-activating protein 1; KO, knockout; KSR, kinase suppressor of RAS; MORG1, MAPK organizer protein 1; N-terminal, amino-terminal; PAK3, p21-activated protein kinase 3; PEBP4, phosphatidylethanolamine-binding protein 4; PKA, protein kinase A; PP, protein phosphatase; RBD, RAS-binding domain; RKIP, RAF kinase inhibitor protein; RTK, receptor Tyr kinase.

An important question tackled in the following years was the role of RAF proteins with respect to other oncoproteins; an important finding was that v-RAF could promote S phase entry of cultured cells even in the absence of RAS activity[28]. This suggested that RAF acts either downstream or in parallel to RAS and hinted at the existence of a mechanism involved in relaying mitogenic signals.

The advent of gene cloning in the 1980s made it possible to search for proto-oncogene homologues across species. Notably, RAF homologues were found in distantly related metazoans such as *Drosophila melanogaster* and *Caenorhabditis elegans*[29–32], and genetic analyses in these organisms confirmed that RAF plays a part downstream of receptor Tyr kinases (RTKs) and RAS in cell proliferation and differentiation events, as inferred by mammalian cell culture experiments.

RAF kinases as core members of the ERK module. The identification of RAF substrates was another major challenge of the late 1980s. Around that time, the discovery of ERK1 and ERK2 established an additional class of cytoplasmic Ser/Thr kinases that transduced proliferative cues[33–35]. ERK1 and ERK2 are activated by dual phosphorylation of conserved Thr and Tyr residues within their activation segment[36,37], an event mediated by either of the two related dual-specificity kinases known as MEK1 and MEK2 (REF. 37) (FIG. 2b). Upon their activation by MEKs, ERK1 and ERK2 were shown to phosphorylate hundreds of substrates, thereby affecting the enzymatic activity, ability to associate with other proteins or with nucleic acids, subcellular localization or stability of these substrates. ERK phosphorylation occurs in several cellular compartments, targeting protein machinery responsible for many basic cellular processes. In addition, a plethora of signalling pathways and gene regulatory circuits are controlled by ERK-dependent phosphorylation. Given its large array of targets, it is not surprising that ERK signalling affects a wide set of cellular events, including growth, proliferation, differentiation, survival and migration (FIG. 2b) (reviewed in REF. 38).



Because their pattern of activation was similar, the biochemical relationship between RAF, MEK and ERK was investigated and resulted in the finding that RAF proteins activate MEK1 and MEK2 by phosphorylating their activation segment (at Ser218 and Ser222 in MEK1)[39] (FIG. 2b). This landmark work identified the first physiological substrates for CRAF and introduced the concept of a MAPK phosphorylation cascade, whereby RAF played the part of a MAPK kinase kinase (MAPKKK). Yet, it was unclear how upstream signals were communicated to the MAPK module. However, correlative observations had linked RAS activity with RAF and ERK activation status[40,41]. The RTK-induced events leading to RAS GTP-loading had been elucidated early in the 1990s[42], but how RAS transmitted those signals to downstream targets such as ERK remained a mystery. Then, in 1993 several groups independently reported that GTP-bound RAS could physically contact RAF, leading to RAF activation[43–46]. These seminal findings provided a unified view as to how signals generated from membrane-bound receptors are conveyed by RAS and then passed on to the three-tiered RAF–MEK–ERK cascade (FIG. 2b). The profound impact that this discovery had on our understanding of signalling mechanisms, and the close ties of this cascade with cancer, made it an eminently scrutinized topic.

KSR proteins are close RAF relatives. Although a coherent picture of the role of RAF and its position in RAS-mediated signalling had emerged by 1993, breaches in our understanding of its activation mechanism started to appear. For instance, reconstituting RAF kinase activity *in vitro* was challenging, which hinted that components other than RAS were likely to regulate its activity[47]. Indeed, heat shock protein 90 (HSP90) and its co-chaperone CDC37, as well as 14-3-3 proteins, were identified soon thereafter as RAF-associated proteins that are crucial for its maturation and activation[48–52]. To identify additional factors contributing to RAS–ERK signalling, genetic screens were carried out in *C. elegans* and *D. melanogaster*. They concomitantly identified loss-of-function alleles in a kinase-encoding gene that suppressed RAS-induced phenotypes and that were thus named kinase suppressor of RAS (*ksr*)[53–55]. Two KSR homologues were found in mammals[55,56], and sequence comparison defined five conserved areas (CA1–CA5) in these proteins (FIG. 2a). CA1 consists of a coiled-coil fused to a sterile α-motif (CC-SAM[57]); CA2 is a conserved Pro-rich stretch of unknown function; CA3 folds as a classical CRD[58]; CA4 is a Ser/Thr-rich region that includes an ERK-binding FXFP motif[59]; and CA5 corresponds to a kinase domain. Importantly, KSR proteins share significant sequence identity with RAF proteins[55] and have a similar domain organization (FIG. 2a). These observations strongly suggest that RAF and KSR isoforms, which we group as RAF family members, arose from a common ancestral gene. Both subfamilies nonetheless have signature domains: the RBD is uniquely found in RAF, whereas KSR proteins contain the two distinctive CA1 (CC-SAM) and CA2 regions.

RAS GTP-loading
The process of loading GTP onto RAS to make it active, which is mediated by guanine nucleotide exchange factors (GEFs). Active GTP-bound RAS reverts to the basal, inactive GDP-bound state by the action of GTPase-activating proteins (GAPs).

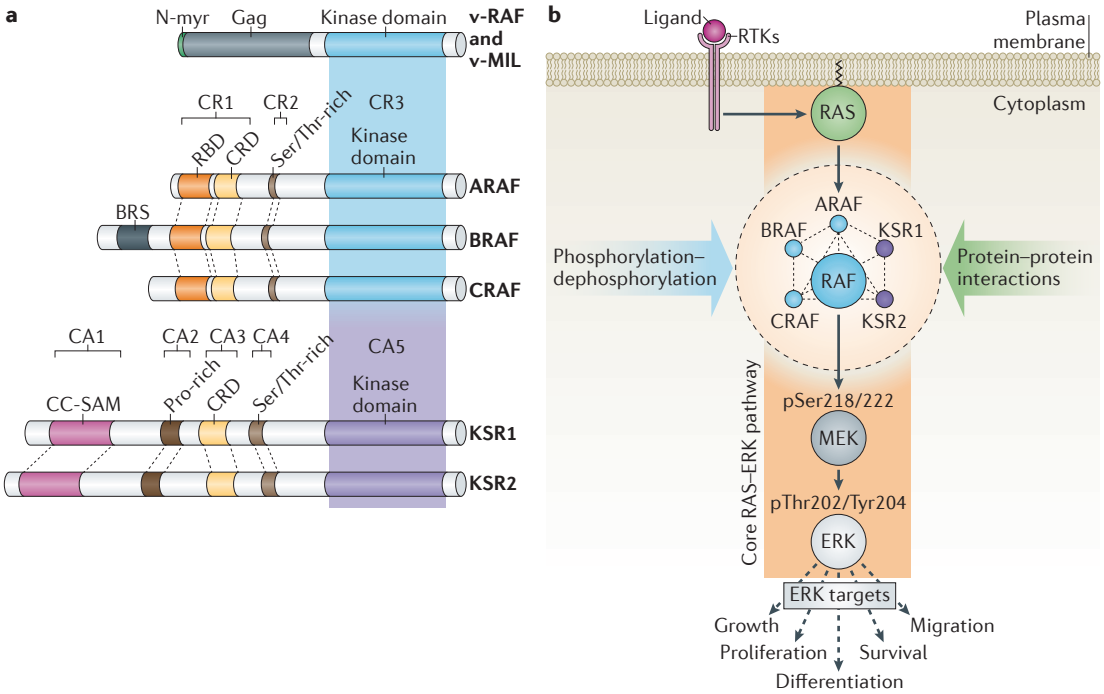


Figure 2 | **RAF and KSR proteins within the RAS–ERK pathway.** **a** | Primary structure highlights the domain architecture of mammalian RAF proteins (ARAF, BRAF and CRAF) and their kinase suppressor of RAS (KSR; KSR1 and KSR2) paralogues. The common structure of the viral oncoproteins v-RAF and v-MIL is also depicted. Viral oncoproteins have amino-terminal truncations compared to their cellular homologues and are fused to the *N*-myristoylated (N-myr) viral Gag protein. RAF proteins are composed of three conserved regions (CR1–CR3), and KSR proteins comprise five conserved areas (CA1–CA5). The schematic shows the relative positions of RAS-binding domain (RBD), Cys-rich domain (CRD), Ser/Thr-rich sequences, kinase domains, BRAF-specific region (BRS)[244], coiled-coil sterile α-motif (CC-SAM) and Pro-rich sequence. **b** | Core components of a typical RAS–ERK signalling cascade are depicted. Incoming signals in the form of ligand-activated receptor Tyr kinases (RTKs) activate RAS. RAF and KSR family members form a network of interacting kinases (dashed lines) that are regulated by activated RAS, as well as by a network of peripheral protein–protein interactions and key phosphorylation–dephosphorylation events. Activated RAF phosphorylates MEK on two key Ser residues (Ser218 and Ser222 in human MEK1, and Ser222 and Ser226 in human MEK2), which in turn activates ERK by causing dual phosphorylation of its activation segment (Thr202 and Tyr204 of human ERK1, and Thr185 and Tyr187 of human ERK2). Phosphorylation sites depicted are those found in MEK1 and ERK1. Upon activation, ERK phosphorylates a wide range of targets that can elicit various cellular responses, including growth, proliferation, differentiation, survival and migration.

In addition to linking it to RAS–ERK signalling, early work on KSR suggested that it acts between RAS and RAF and, therefore, might be involved in RAF activation[55]. In agreement with this, recent work showed that KSR proteins function as allosteric inducers of RAF catalytic function[60] (FIG. 2b). KSR proteins were also found to bridge various components of the pathway, in particular RAF and MEK[61–63], which suggests that they also serve as scaffolding factors orchestrating RAS–ERK signalling. Although KSR proteins have a kinase domain, studies demonstrating both the presence[64–67] and the absence[62,63,68] of KSR kinase activity have been published, and whether KSRs are catalytically competent enzymes remains an ongoing debate.

The RAF activation cycle

Once RAF proteins had been positioned in the RAS–ERK pathway, several laboratories embarked on characterizing their complex regulation. This led to the identification of various proteins and post-translational modifications that impose positive or negative constraints onto RAF (FIG. 2b). These inputs trigger conformational changes that enable the transition from a quiescent to a catalytically competent enzyme and then a return to the basal state. This series of events can be depicted as a cycle with defined steps (FIG. 3a).

RAF in quiescent cells. Inactive RAF exists in an autoinhibited state whereby its N-terminal region contacts and represses its catalytic domain (FIG. 3a). Three observations are consistent with this model: deletion of the RAF N-terminal regulatory sequences results in an unregulated kinase[12–20]; overexpression of the CR1 domain (minimally containing the RBD and CRD) suppresses RAF kinase activity in *trans*[69–72]; and the CR1 associates with the kinase domain[69–72]. However, the structural interplay between the N-terminal region and the kinase domain of RAF has yet to be documented.

Phosphorylation of the CRAF CR2 at Ser259 (or of the equivalent site, Ser365, in BRAF) also helps to suppress RAF catalytic activity. Protein kinase A (PKA)[73–75] and AKT[76,77] are the main kinases phosphorylating this site. LATS1 was also recently found to phosphorylate this site as part of a crosstalk with the MST2–Hippo

signalling pathway[78]. A negative function for phosphorylated Ser259 is supported by experiments in which this Ser is substituted with Ala[75,79] as well as by gain-of-function mutations in tumours and RASopathies that target this residue or amino acids adjacent to it (see below). These genetic alterations systematically increase RAS–RAF binding and the recruitment of RAF to the plasma membrane, which drives RAF activation[80,81].

The mechanism by which phosphorylated Ser259 inhibits CRAF activity was uncovered when it was found to be a phosphorylation-dependent binding motif for 14-3-3 proteins[82–85] (FIG. 3a). RAF proteins contain at least two binding sites for 14-3-3 proteins[49]. In CRAF, the second site encompasses phosphorylated Ser621, which is situated near its carboxyl terminus[82–84] (FIG. 3a). The simultaneous binding of 14-3-3 to phosphorylated Ser259 and phosphorylated Ser621 within a single RAF protein is often portrayed as stabilizing the inhibitory interaction between the N-terminal region and the kinase domain[1,85]; however, there is no direct evidence supporting this model. Interestingly, a cryptic 14-3-3 binding site centred on phosphorylated Ser233 was found to synergize with phosphorylated Ser259 in engaging dimeric 14-3-3 proteins[86,87]. Whether this binding mode, which is restricted to the N-terminal region, suffices to suppress CRAF has not been addressed.

Recruiting RAF to the plasma membrane. The recruitment of RAF to the plasma membrane, which is initiated by GTP-loaded RAS, is an essential step in its activation and depends on the concerted action of the RAF RBD and CRD (FIGS 2a,3). The RBD is sufficient for the interaction of RAF with the effector loop of the three main RAS isoforms, HRAS, KRAS and NRAS, which are themselves anchored to the plasma membrane via prenylation of their C-terminal CAAX BOX[88]. The RAF RBD consists of a ubiquitin fold that is structurally analogous to RBDs found in other RAS effectors, including PI3K p110 subunits and RAL guanine nucleotide dissociation stimulator (RALGDS)[89–91]. The RBD is essential for normal RAS–ERK pathway signalling, and single amino acid substitutions that disrupt the association of RBD with RAS abolish RAF membrane recruitment and activation[92]. In addition to the three classical RAS proteins, other small GTPases of the RAS family interact with RAF and, when overexpressed, modulate ERK signalling in sometimes opposite manners[93–96]. It remains possible that these GTPases indirectly affect RAF, as they are all involved in other signalling cascades.

CRDs form zinc-coordinated structures that interact with phospholipids and thereby play a part in the membrane translocation and activation of several kinases[97]. The CRD of CRAF directly interacts with phosphatidylserine[98,99] and is crucial for stably linking CRAF proteins to the plasma membrane[100,101]. In addition to phospholipids, RAF CRDs selectively interact with farnesyl groups attached to the C-terminus of RAS proteins[102–106]. This might explain why farnesylation increases the association of RAS with full-length RAF *in vitro*[102]. Thus, the RBD and CRD of RAF can both interact with RAS, albeit with distinct sites. This observation, together with their tandem arrangement, suggests that the two domains form a structural and functional entity that deserves more detailed study.

Even though the RAS–RAF connection was established more than 20 years ago, the structural changes that RAS imposes on RAF remain elusive. For example, it is unclear how RAS disrupts the auto-inhibitory interaction between the CR1 and the kinase domain of RAF[72]. RAS activity also promotes the dephosphorylation of the inhibitory 14-3-3 binding site in the N-terminal region of RAF[80]. Both protein phosphatase 2A (PP2A) and PP1 participate in this event[80,107–109], which releases 14-3-3 from the RAF N-terminus and thereby promotes the anchoring of RAF proteins to the plasma membrane via their CRDs (FIG. 3a). The mechanism by which RAS triggers these events and the structural consequences of recruiting RAF to the plasma membrane have yet to be delineated.

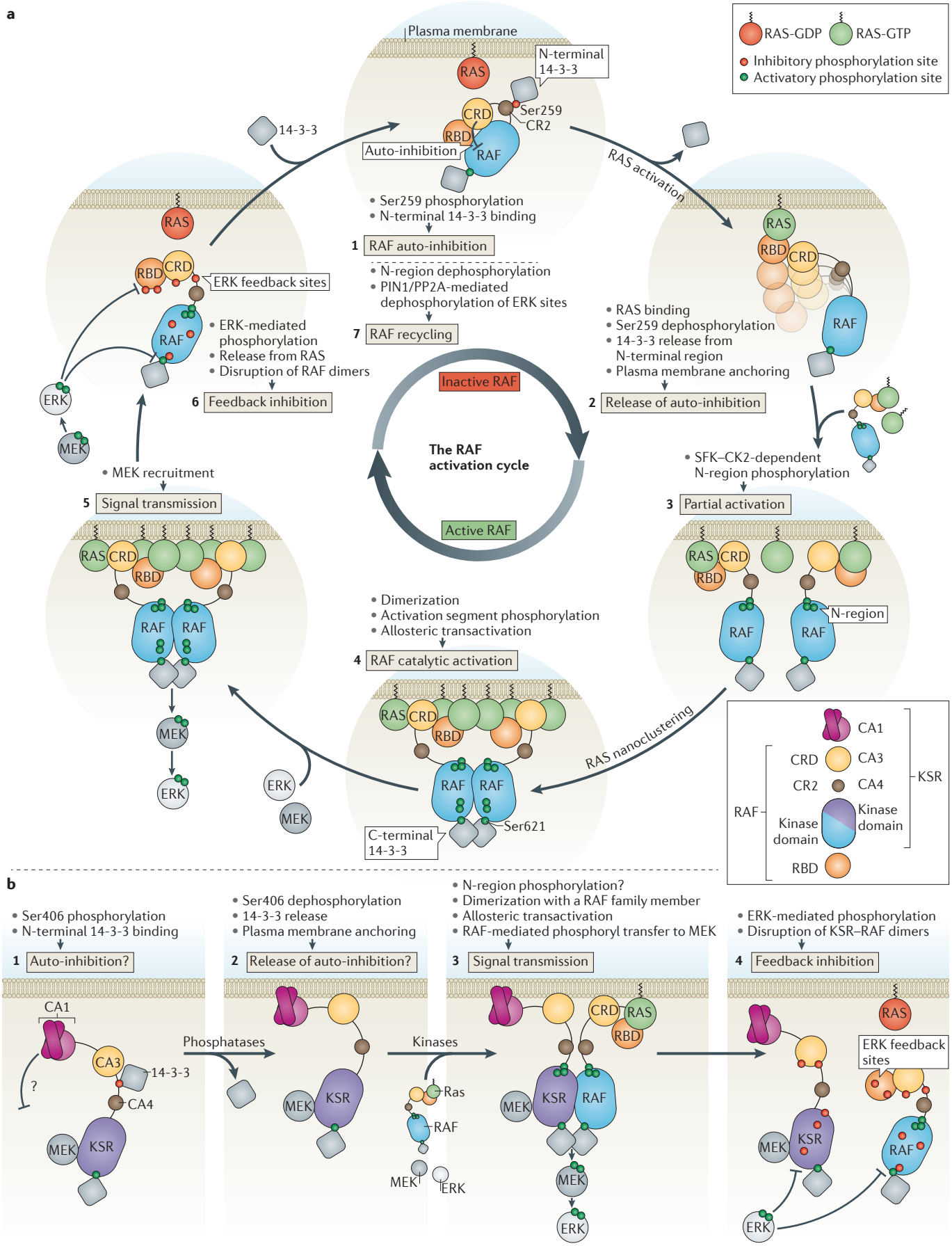
Activation of RAF by dimerization. More recent data show that dimerization of the RAF kinase domain is a central RAS-regulated event in RAF activation. The first evidence for this came from the observation that artificial oligomerization of RAF stimulated its activity[110,111]. The physiological importance of this finding became clear when RAS was found to promote the formation of active BRAF–CRAF complexes[112,113], and the proteins encoded by numerous catalytically impaired oncogenic *BRAF* alleles were shown to promote ERK signalling by their enhanced ability to associate with endogenous CRAF[114–116]. This suggested that BRAF could induce the catalytic function of CRAF independently of the intrinsic kinase activity of BRAF. In addition to BRAF–CRAF heterodimers, the respective homodimers were found to form under physiological conditions, but their activity was lower than that of the heterodimers[113]; this suggests that RAF activity emanates from a variety of oligomers.

In parallel to these studies, RAF activation in *D. melanogaster* was shown to depend on the kinase domain, but not the putative kinase activity, of KSR[117]. A genetic screen to isolate modifiers of a phenotype caused by activated RAS in *C. elegans* had previously identified a loss-of-function allele targeting a residue remote from the presumed catalytic site of KSR-1 (Arg531His)[54]. Intriguingly, this mutation abrogated the ability of KSR to transactivate RAF in *D. melanogaster*[117]. Analysis of publicly available human BRAF kinase domain crystals led to two notable observations. First, the asymmetrical unit of these crystals comprised two kinase domains contacting each other through a side-to-side interface that is highly conserved across RAF and KSR family members[60] (FIG. 4a). Second, BRAF Arg509 (equivalent to Arg531 in *C. elegans* KSR-1) formed the heart of this interface, participating in an extended network of hydrogen bonds between the two protomers (FIG. 4a). Importantly, amino acid substitutions of Arg509 (in particular, Arg509His, which mimics the Arg531His mutation in *C. elegans*) or of any other conserved residue in the side-to-side interface impeded RAF dimerization and activity[60]. This work established that both RAF and KSR family members can form physiologically relevant side-to-side homodimers and heterodimers, resulting in protomer transactivation.

RASopathies
A group of developmental diseases — including neurofibromatosis type 1, Legius syndrome, Costello syndrome, cardio-facio-cutaneous syndrome, LEOPARD syndrome and Noonan syndrome — that share a common set of clinical manifestations. They are characterized by germline gain-of-function mutations in various genes encoding components of the RAS–ERK pathway. So far, only cardio-facio-cutaneous, LEOPARD and Noonan syndromes have been associated with mutations in *CRAF* or *BRAF*.

Prenylation
Post-translational addition of a lipidic polyisoprenyl group (either a 15-carbon long farnesyl or a 20-carbon long geranylgeranyl moiety) on the Cys residue of a CAAX box. Prenylation of proteins facilitates their association with cellular membranes.

CAAX box
Sequence motif found at the carboxyl terminal end of most small GTPases and also in other types of proteins. C stands for Cys, and A for an aliphatic residue; X represents any amino acid. A Leu residue at the X position dictates geranylgeranylation, whereas other residues specify farnesylation.



The mechanism leading to dimerization-dependent RAF activation is only partially understood, but it is likely to involve allosteric control of structural elements of the catalytic cleft. Generally, protein kinase domains comprise an N-lobe of five antiparallel β-strands and a regulatory helix denoted αC. The N-lobe is connected by a flexible hinge to the larger C-lobe, which predominantly consists of α-helices and includes a key loop termed the activation segment (FIG. 4a). Although inactive kinases oscillate between open and closed conformations through inter-lobe motions[118], the active state is restricted to the closed conformation, in which catalytic amino acids assume an orientation permissive for catalysis (FIG. 4b). This closed arrangement is held in place by the alignment of two parallel columns of spatially conserved hydrophobic residues spanning the N- and C-lobes, termed the catalytic (C-) and regulatory (R-) spines, respectively (FIG. 4b). In-depth comparison of inactive and active states of several kinases showed that a series of recurring conformational changes, controlled by protein–protein interactions and phosphorylation of the activation segment, lead to spine alignment, kinase closure and activation[119,120]. The most notable of these motions is the inward movement of the αC helix and the 'in' positioning of a Phe residue that is part of the highly conserved DFG motif, which is situated at the N-terminal base of the activation segment (FIG. 4b). In RAF, the monomer-to-dimer transition is thought to globally stabilize the closed conformation of the kinase domain by restraining inter-lobe motions and by promoting key conformational transitions[121] (FIG. 4c). In particular, given that the C-terminal tip of the RAF αC helix encompasses the Arg509 residue, which forms the core of the side-to-side dimerization interface, it is postulated that this helix assumes a productive 'in' conformation upon dimerization[60] (FIG. 4c).

This was recently corroborated by the first monomeric structure of the BRAF kinase domain[122]. One salient feature of this structure that is not present in RAF dimeric crystals is the presence of a short inhibitory helix within the activation segment (activation segment helix 1) (FIG. 4c). This helical element establishes numerous contacts with the catalytic site and forms an extended hydrophobic network with the αC helix, thereby stabilizing its inactive 'out' position (FIG. 4c). This finding provides insights into how the αC helix motions and the dimer interface are allosterically coupled with conformational changes of the activation segment. However, this model is based on static crystal structures, and dynamic biophysical assays are needed to probe the underlying conformational transitions. Another question is whether RAF kinase domain dimerization is invariably required to attain the active state under physiological circumstances. Indeed, recent evidence, gained in part using a BRAF oncogenic variant with the Val600Glu mutation, suggests that RAF activity and ERK signalling do not always correlate with RAF dimerization[123–125]. However, this conclusion needs to be substantiated under physio-logical conditions.

The BRAF side-to-side interface exhibits a low affinity *in vitro*[60,121]; thus, cofactors, other parts of RAF and/or post-translational modifications might modulate dimerization. Indeed, RAS has been shown to induce RAF dimerization[112]. The underlying mechanism is not clear, but a RAS-mediated increase in the concentration of RAF in the 2D space of the plasma membrane and relief of RAF auto-inhibition by its N-terminal domain might contribute to this event. In addition, RAS forms nanoclusters at the plasma membrane[126,127] and, similarly to RAF, GTP-loaded RAS can form dimers *in vitro* and *in vivo*[128–130] (FIG. 3a). It is thus possible that membrane-attached dimeric RAS directly contributes to RAF activation by coupling two RAF molecules. Interestingly, RAS is also required for RAF dimerization that is artificially induced by ATP-competitive inhibitors[116,131,132], and inhibitor-induced RAF dimerization reciprocally promotes RAS nanoclustering at the plasma membrane[133]; this suggests that the two events function cooperatively. Upon interaction with RAS, another factor that might influence RAF dimerization is the membrane micro-environment itself, including phospholipids[99,134].

Finally, the binding of 14-3-3 proteins to the C-terminal tail of RAF has also been found to enable its dimerization and transactivation[113,115]. The mechanism is unclear, but it is hypothesized that dimeric 14-3-3 complexes bind to CRAF on phosphorylated Ser621 or to BRAF on phosphorylated Ser729 and thereby stabilize the interaction between two RAF protomers[60] (FIG. 3a).

◀ Figure 3 | **The RAF activation cycle.** The main events and steps governing RAF and kinase suppressor of RAS (KSR) activation are depicted. Human CRAF and KSR1 are used as reference. **a** | Step 1: Auto-inhibited RAF proteins stabilized by 14-3-3 proteins bound to phosphorylated Ser259 at the RAF amino terminus are found in the cytosol. Step 2: Upon stimulation by mitogens, GTP-loaded RAS directly interacts with RAF proteins through their RAS-binding domains (RBDs), thereby initiating plasma membrane recruitment of RAF. The interaction with RAS relieves RAF auto-inhibition and correlates with Ser259 dephosphorylation and release of inhibitory 14-3-3. Step 3: Membrane recruitment brings RAF in contact with kinases, including SRC family kinases (SFKs) and casein kinase 2 (CK2), which phosphorylate a series of activating sites in the negatively charged (N-) region. Step 4: Membrane binding and RAS nanoclustering augment the effective concentration of RAF and thereby contribute to RAF dimerization. 14-3-3 proteins bound to phosphorylated Ser621 at the carboxy-terminal tail also participate in RAF kinase domain dimerization. Step 5: Dimerization induces RAF catalytic activity, thereby enabling MEK recruitment and phosphorylation, and signalling down the three-tiered MAPK module. Step 6: ERK signalling implements a negative feedback loop in which ERK phosphorylates several inhibitory sites in distinct regions of activated RAF, causing a release from activated RAS and the disruption of RAF dimers. Step 7: Negative feedback is coordinated with dephosphorylation of RAF-activating phosphorylation sites as well as ERK-targeted phosphorylation sites; hence, RAF proteins are recycled for later rounds of activation. Note that 14-3-3 binding to the RAF carboxy-terminus is depicted as constitutive, but recent evidence suggests the possibility of regulation. **b** | Steps involved in KSR regulation often parallel those defined for RAF proteins. Step 1: Inactive KSR proteins are kept in the cytosol through interaction with inhibitory 14-3-3 proteins in their N-terminal region. KSR and MEK proteins form constitutive complexes. Step 2: the dephosphorylation of Ser406 allows 14-3-3 release and plasma membrane anchoring of KSR proteins via conserved area 1 (CA1) and CA3. Step 3: KSR proteins heterodimerize with other RAF proteins, leading to RAF transactivation and MEK–ERK signalling. Step 4: ERK-mediated negative feedback phosphorylation of several sites in RAF and KSR disrupts RAF–KSR dimers, leading to signal attenuation. Whether the N-terminal region of KSR negatively controls the kinase domain is unknown (as indicated by the question mark). Similarly, whether the KSR N-region is phosphorylated and contributes to activation remains unknown. CR, conserved region; CRD, Cys-rich domain; PIN1, peptidyl-prolyl *cis–trans* isomerase NIMA-interacting 1; RBD, RAS-binding domain.

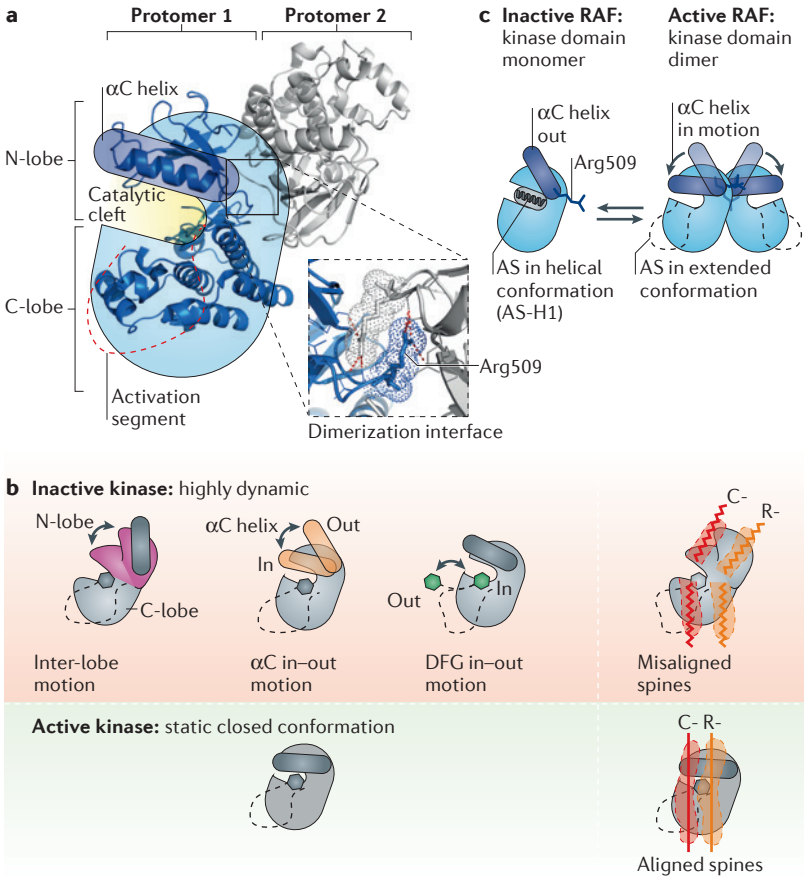


Figure 4 | **Regulation of the RAF kinase domain by dimerization.** **a** | Most RAF kinase domain crystal structures consist of two protomers (here exemplified by BRAF Protein Data Bank identifier (PDB) 1UWH[114]) forming a dimeric assembly through a conserved side-to-side dimerization interface centred on Arg509 (enlarged area). The blue oval indicates the surface covered by the kinase domain. **b** | Main conformational transitions between inactive and active states of a prototypical kinase domain are shown. The conformational flexibility of inactive kinases (top) does not allow the alignment of two parallel columns of spatially conserved hydrophobic residues, termed the regulatory (R-) and catalytic (C-) spines (top right), thereby precluding catalytic activation. At least three distinct motions were documented for kinase domains: inter-lobe motion, αC helix 'in–out' motion and DFG motif in–out motion. By contrast, active kinases (bottom) display an overall static 'closed' conformation that leads to the alignment of hydrophobic spines, kinase closure and activation (bottom right). **c** | The monomer-to-dimer transition of RAF proteins is thought to restrict kinase domain dynamics and to stabilize the closed state, thereby leading to catalytic activation. One of the conformational changes induced by dimerization is the positioning of the αC helix in its 'in' conformation, which leads to a productive alignment of the hydrophobic spines and of the main residues involved in catalysis. In the monomeric state, the αC helix is maintained in its 'out' conformation by a structured portion of the activation segment (AS) that forms a short helix (AS helix 1 (AS-H1); left). Upon dimerization, the AS is thought to adopt an extended conformation (right), allowing the αC helix to move inward. The carboxy-terminal tip of the αC helix, comprising Arg509, is part of the dimerization interface, suggesting that the dimerization surface, the αC helix and the AS are allosterically coupled.

Activation of RAF by phosphorylation. RAF proteins undergo numerous phosphorylation events throughout their activation cycle (FIG. 5). Analysis of phosphorylation proteomic data sets[135] provides a 'snapshot' of the extent of these modifications (see Supplementary information S1 (figure)). Unsurprisingly, given the size of the human kinome and the degenerate nature of kinase consensus sites, it was difficult to connect RAF phosphorylation sites to their cognate kinases and to identify the consequences of these phosphorylation events. Furthermore, it took several years to untangle the events that promote RAF activity from those that turn it off (for example, phosphorylation of Ser259) (FIG. 5a). Three main areas within RAF require phosphorylation for catalytic activation: the negatively charged (N-) region, the activation segment and the C-terminal 14-3-3 binding site (FIG. 5b–d).

The N-region minimally consists of a stretch of four residues situated immediately N-terminal to the kinase domain (see Supplementary information S1 (figure)). Its primary sequence differs between RAF family members, allowing divergent regulation[136] (FIG. 5b, left panel). For example, in CRAF the SSYY submotif (residues 338–341) requires both Ser and Tyr phosphorylation for full activity, and phosphorylation of Ser338 and Tyr341 in particular are critical[137]. By contrast, the homologous SSDD submotif (residues 446–449) in BRAF only allows Ser phosphorylation, but the presence of Asp448 and Asp449 readily provides the negative charges necessary for activity[138,139]. SRC family kinases (for example, SRC, LCK and FYN) seem to be the main Tyr kinases targeting the N-region of both ARAF and CRAF[140–143]. With respect to Ser338 in CRAF, p21-activated protein kinase (PAK) family members were the first candidates linked to its phosphorylation as part of a PI3K–CDC42 or RAC signalling axis[144–146], but this connection has been disputed[147]. More recently, casein kinase 2 (CK2) was also found to phosphorylate CRAF at Ser338 and the homologous residue, Ser446, in BRAF[148]. CK2 is a constitutively active enzyme with the consensus phosphorylation site S/TXXD/E, in which the +3 position (D/E) can also be a phosphorylated Ser, Thr or Tyr residue. This provided an explanation as to why the CK2-mediated phosphorylation status of the BRAF N-region does not seem to be regulated, whereas both ARAF and CRAF require stimulus-dependent Tyr phosphorylation before CK2 phosphorylation[148].

Although functionally critical, the structural role of the N-region phosphorylation status is not fully understood. However, recent work suggested that it could determine the transactivating direction in RAF dimers[149] and thus impose an asymmetry that is reminiscent of the mechanism leading to epidermal growth factor receptor (EGFR) allosteric activation[150]. Indeed, kinase-impaired RAF molecules harbouring negatively charged phosphomimetic residues in their N-region acted as either 'activator' or 'receiver' units, whereas non-phosphorylated (or uncharged) variants could behave only as receiver kinases[149] (FIG. 5b, right panel). These findings could explain why BRAF, with its acidic N-region, has a higher basal activity than ARAF or CRAF. Although more structural and functional characterization is needed to fully appreciate this mechanism, it seems that N-region phosphorylation and kinase domain dimerization both impinge on kinase domain closure[149] (FIG. 5b), which is required for catalytic switching in most protein kinases[119].

Like several other kinases, RAF activity also seems to require phosphorylation of its activation segment. Two conserved residues in this region are functionally relevant

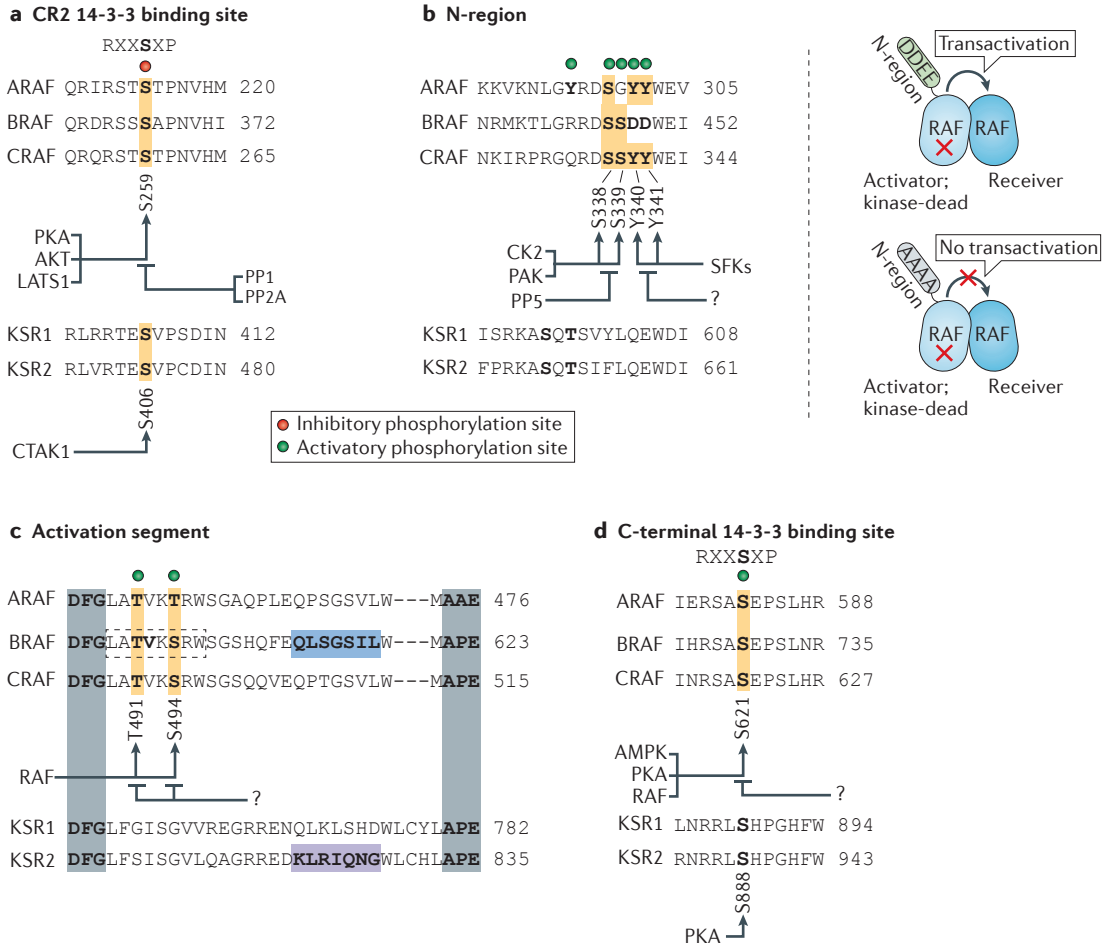


Figure 5 | **Regulation of RAF and KSR proteins by phosphorylation.** Amino acid sequence alignment of the key regulatory areas controlled by phosphorylation in RAF and kinase suppressor of RAS (KSR) proteins is shown. Kinases (arrows) and phosphatases (blunted lines) acting on highlighted residues are indicated. Positively and negatively acting phosphorylation sites (also see Supplementary information S1 (figure)) are indicated by green and red circles, respectively. Phosphorylation sites that have been experimentally validated are highlighted in yellow. **a** | Conserved region 2 (CR2) comprises a central phosphorylated Ser (S259 in CRAF) that is bound by inhibitory 14-3-3 proteins. 14-3-3 consensus sites are defined by a RXXS/TXP motif, where X represents any amino acid. **b** | The negatively charged (N-) region comprises several phosphorylation sites that differ between RAF family members. The right panel schematically depicts the importance of the negative charge in the N-region in dictating asymmetrical transactivation of RAF dimers. Specifically, RAF molecules harbouring negatively charged phosphomimetic residues (DDEE) in their N-region act as 'activator' or 'receiver' units, whereas non-phosphorylated (or uncharged) variants (AAAA) can behave only as receiver kinases. **c** | The activation segment of RAF proteins comprises two residues (T491 and S494 in CRAF) that are modified by as yet unknown kinases and phosphatases. The highly conserved DFG and APE motifs at each end of the activation segment are shown with grey shading. A dashed box surrounds the sequence element that forms the inhibitory activation segment helix 1 (AS-H1) in monomeric BRAF. The regions of contact between BRAF or KSR2 and the activation segment of MEK1 are highlighted in blue and purple, respectively. The V600 residue (bold) is frequently mutated in oncogenic BRAF. **d** | The 14-3-3 binding site found in the carboxy-terminal tail of the kinase domain is common to all RAF and KSR family members. **Question marks indicate** that kinases and phosphatases targeting the specified sites are unknown. AMPK, AMP-activated protein kinase; CK2, casein kinase 2; PAK, p21-activated protein kinase; PKA, protein kinase A; PP5, protein phosphatase 5; SFK, SRC family kinases.

for catalysis in human ARAF (Thr452 and Thr455)[151], BRAF (Thr599 and Ser602)[152] and CRAF (Thr491 and Ser494)[153] (FIG. 5c). Nevertheless, detecting these phosphorylation events remains challenging. For instance, they have not yet been retrieved in large-scale phosphoproteomic studies, in contrast with the abundant detection of several other phosphorylation sites (see Supplementary information S1 (figure)). This might be explained by their low levels or their transient nature, or because of technical limitations. Moreover, as the activation segment is systematically dephosphorylated and mostly unstructured in RAF crystals, it is currently not possible to decipher the conformational consequence of its phosphorylation. It also remains unclear which kinase is responsible for this phosphorylation event, although a recent study using an *in vitro* kinase assay suggested that *cis* autophosphorylation was at play[149]. The physiological importance of these findings has yet to be determined.

The functional relevance of 14-3-3 protein recruitment on the RAF C-terminal tail has been discussed above. In addition to stabilizing RAF dimerization, 14-3-3 proteins bound to the sequence centred on phosphorylated Ser621 (FIG. 5d) seem to stimulate binding of RAF to ATP, subsequently leading to MEK phosphorylation[154]. Although the mechanistic importance of this 14-3-3-binding site is broadly recognized, its precise influence on RAF activity is not understood and may also entail a negative role depending on the context[155,156]. Moreover, the identity of the kinase responsible for Ser621 phosphorylation is not resolved. There is some evidence for autophosphorylation[157], but other kinases have also been proposed to target this site[155,156,158], although their relative importance remains to be established. Finally, whether the phosphorylation of this site is a controlled or constitutive event has been a lingering question. Although the details are still uncertain, recent findings are consistent with a regulation through AMP-activated protein kinase (AMPK) signalling[156].

The role of KSR in RAS–ERK signalling. As is typically the case for signal transduction pathways, the diverse cell-specific processes controlled by RAS–ERK signalling depend on the precise subcellular distribution and timely assembly of higher-order complexes comprising core pathway components. Although they can interact with each other through dedicated interfaces, RAS–ERK pathway members also rely on scaffolding proteins, including KSR[159], CNK (connector enhancer of KSR)[160], SUR-8 (in *C. elegans*; known as SHOC2 in humans)[161], β-arrestin[162], paxillin[163], MORG1 (MAPK organizer protein 1)[164], AKAP13 (A-kinase anchor protein 13; also known as AKAP-LBC)[165] and IQGAP1 (IQ motif containing GTPase-activating protein 1)[166]. These proteins physically bridge RAF to other RAS–ERK pathway core components or to other signalling molecules, thereby enabling pathway crosstalk. They have been reviewed elsewhere[167] and, except for KSRs, are not discussed here.

Scaffolding proteins are typically depicted as non-catalytic polypeptides that passively tether enzymes to their substrates. However, in the past few years, indications that scaffolds can allosterically control specific catalytic events[168] have expanded their functional repertoire. In the case of RAF, only KSR proteins have so far been suggested to implement an allosteric step[60]. KSR proteins were originally defined as RAS–ERK scaffolding proteins because of their capacity to bring RAF, MEK and ERK together in a dose-dependent manner that correlates with their ability to sustain ERK signalling in different biological contexts[61–63,159,169,170]. The identification of additional regulators, such as CK2 and PP2A, that tightly interact with KSR proteins further reinforced the notion that KSR proteins nucleate large protein assemblies that regulate RAF[109,148]. However, the concept that KSR proteins work as scaffolds for the three core constituents of the ERK module has not been formally demonstrated. Indeed, there is no indication that KSR-bound RAF can phosphorylate MEK bound to the same KSR molecule, and recent structural data suggest that this is not topologically possible (see below). Moreover, KSR proteins seem to associate only with activated ERK in part to implement a negative feedback loop[171], which indicates that this interaction is not required for signal transmission from MEK to ERK. Finally, the fact that KSRs heterodimerize with RAFs and allosterically stimulate their catalytic activity[60] suggests that they are not merely passive scaffolds.

Moreover, KSR proteins are subjected to a complex regulation that parallels steps of the RAF activation cycle (FIG. 3b). Their regulation entails: an inhibited state in the cytosol, which is stabilized by 14-3-3 binding to specific sites in the N-terminal region that are phosphorylated by CTAK1 (also known as MARK3)[172] (FIG. 5a; see Supplementary information S1 (figure)); PP2A-dependent release of 14-3-3 from these motifs[109,172,173], leading to the re-localization of KSR to the plasma membrane by a process engaging their CA1 (REF. 57) and CA3 (REF. 58) domains; formation of homodimers and heterodimers with other RAF or KSR family members through a shared side-to-side interface[60,64,174]; and phosphorylation on multiple sites as part of an ERK-dependent negative feedback loop which, in turn, leads to the release of KSR from the plasma membrane and abrogation of dimerization[171]. KSR proteins also contain potential regulatory phosphorylation sites in their N-region, activation segment and C-terminal 14-3-3 binding site (FIG. 5; see Supplementary information S1 (figure)) that are likely to be involved in controlling their dimerization and activity, similarly to RAF proteins. Recent discoveries have highlighted other scaffolding functions of KSR proteins that, through a phosphorylation-based modulation of their N- and C-terminal 14-3-3-binding sites, implement crosstalk between RAS–ERK, calcium–calcineurin and PKA signalling[165,175].

Binding of MEK to RAF and KSR complexes. At some point during the activation cycle, MEK molecules must be recruited to RAF directly or through the bridging action of a scaffolding protein (FIG. 3a). Little structural information on this event was available until recently, when the crystal structures of BRAF–MEK1 and KSR2–MEK1 were solved and found to form heterotetramers, whereby BRAF or KSR2 homodimers associate with two MEK1 molecules[64,176]. The resulting topology of these complexes is MEK1–BRAF–BRAF–MEK1 and MEK1–KSR2–KSR2–MEK1. Interestingly, BRAF and KSR2 interact with MEK1 in a similar face-to-face manner through two main contact points. The first one is their respective αG helices, a structural element frequently implicated in kinase–substrate docking interactions[177]. The other contact involves their respective activation segments, which form an antiparallel β-sheet. An important distinction between the two complexes is that although the BRAF activation segment adopts an active-like extended conformation concomitant with the αC helix in its active 'in' position, the activation segment and αC helix of KSR2 adopt non-productive conformations[64,176]. Another difference is that KSR2 forms homodimers that engage along the same side-to-side interface seen in BRAF and CRAF homodimers, but their orientation is rotated by 180° with respect to the surface plane.

MEK1 phosphorylation was not identified in these two structures[64,176]. Within the BRAF–MEK1 crystal, this is explained by the presence of a MEK allosteric inhibitor that traps MEK1 in a pre-activation state in which MEK1 Ser218 and Ser222 cannot access the RAF catalytic centre. In the KSR2–MEK1 complex, the outward position of the KSR2 αC helix explains the lack of MEK phosphorylation, as it precludes any possibility of phosphoryl transfer. Whether the atypical orientation of the KSR2 protomers, which are rotated by 180°, helps to place the αC helix in this inactive conformation is not known.

Another feature that could be responsible for the lack of MEK1 phosphorylation is the stable β-sheet that forms between the activation segments of KSR2 and MEK1 even in the absence of a MEK inhibitor. This arrangement essentially sequesters the MEK1 activation segment in a non-phosphorylatable state. Given that KSR2 does not seem to phosphorylate MEK1 at Ser218 and Ser222 *in vitro*[64], it has been postulated that an active RAF molecule is recruited to the KSR2–MEK1 complex and phosphorylates MEK. For this model to hold together, two assumptions need to be fulfilled. First, a regulatory event must free the MEK1 activation segment; this event might be the switch from a KSR2–KSR2 homodimer to a RAF–KSR2 heterodimer[64]. Second, the RAF molecule phosphorylating MEK1 must be distinct from the one engaged in the RAF–KSR2 heterodimer, the catalytic cleft of which points in the opposite direction and is topologically unable to reach the activation segment of KSR2-bound MEK1. Therefore, assuming that the structure that forms under crystallographic conditions is representative of physiological complexes, KSR2-bound MEK1 could only be phosphorylated in *trans* by an active RAF molecule from another active dimer[64]. This model has yet to be validated.

Feedback inhibition and return to the inactive state. Signal attenuation and reversion to the basal state are crucial for most RAS–ERK-dependent biological processes. To return to the basal state, positively acting phosphorylated residues on RAF proteins need to be dephosphorylated. However, only Ser338 dephosphorylation by PP5 has been described[178], which leaves room for future investigations. Another key mechanism targeting activated RAF proteins is direct ERK-mediated feedback phosphorylation on several specific residues[179,180] (FIG. 3a). Phosphorylation of some of these sites on BRAF was shown to inhibit RAS binding and to disrupt BRAF–CRAF heterodimers[113,180]. However, this negative feedback loop is futile against specific oncogenic BRAF mutants, such as BRAF-Val600Glu, as they do not require RAS signalling or dimerization for catalytic function. These ERK target sites subsequently need to be dephosphorylated by PP2A in a manner dependent on PIN1 (peptidyl-prolyl *cis–trans* isomerase NIMA-interacting 1), enabling RAF proteins to be recycled for a new round of activation[179] (FIG. 3a).

Dysfunction of RAF proteins in disease

Early work on *RAF* proto-oncogenes hinted at their involvement in human cancer, and studies in animal models repeatedly confirmed this view (BOX 1). However, definitive proof came in 2002, when *BRAF* oncogenic mutations were detected in a wide variety of tumour types with some — for example, metastatic melanoma and papillary thyroid carcinoma — displaying especially high frequencies of *BRAF* alterations (>50%)[181,182] (FIG. 6a,b). Oncogenic *CRAF* mutations have also been recovered but at a much lower rate[183]. Mutations in *ARAF*, *KSR1* and *KSR2* are even rarer in human tumours[183], and their significance is currently unknown, although experimental data in cell lines and mouse models (BOX 1) suggest that they contribute to tumorigenesis.

Besides cancer, causal germline mutations in *BRAF* and *CRAF* are associated with a series of human disorders known as RASopathies (FIG. 6a). Noonan syndrome, LEOPARD syndrome and cardio-facio-cutaneous syndrome belong to this group of diseases[184–188]. As they all have a common aetiology involving increased RAS–ERK signalling, these diseases present overlapping phenotypic features that generally include a predisposition to malignancies[188].

Oncogenic mutations in BRAF or CRAF occur in specific areas of the proteins (FIG. 6a). Although their mechanism of action is not thoroughly understood, recent advances allow a classification based on the RAF activation step that they interfere with or promote. We present oncogenic mutations in BRAF or CRAF according to their order of appearance along the RAF activation cycle (FIG. 3a). These alterations essentially act by releasing inhibition mediated by the N-terminal region, enhancing dimerization of the kinase domain and mimicking phosphorylation of the activation segment.

Releasing inhibition mediated by the N-terminal region. Destabilization of the inhibitory interaction between the N-terminal region and the kinase domain of RAF is arguably the earliest event of the RAF activation cycle targeted by disease-causing mutations in *RAF* genes. N-terminal truncations of CR1 and CR2 domains shaped the early history of *RAF* oncogenes (see above). Consistent with these initial findings and owing to the wealth of tumour sequencing data, the past few years have seen the emergence of a variety of BRAF and CRAF N-terminal truncation and fusion events in distinct cancer types[189–193]. Generally, these fusions arise from chromosomal rearrangements that combine the kinase domain of BRAF or CRAF with the N-terminal portion of a distinct gene product[189–193]. The concerted deletion of CR1- and CR2-encoding exons generates fusion proteins that lack the N-terminal auto-inhibitory sequences, leading to de-repressed RAF activity. In several instances, N-terminal sequences provided by the RAF fusion partner comprise dimer-promoting domains, which are thought to further induce kinase activity[193]. More work is required to fully appreciate how these heterologous N-terminal sequences contribute to RAF dysregulation.

Mutations specifically targeting the CR1 or CR2 domain can also lead to de-repression of RAF activity. Point mutations in the CRD have been found in samples from patients with cancer, albeit at a low frequency[183] (FIG. 6a). It is interesting to note that mutations in the

Noonan syndrome
A genetic disorder that prevents normal development in various parts of the body; clinical presentation includes unusual facial characteristics, short stature and heart defects. Mutations in Tyr protein phosphatase non-receptor type 11 (*PTPN11*), *CBL*, *SOS1*, *RASA2*, *SHOC2*, *KRAS*, *NRAS*, *RIT1*, *CRAF* and *MEK1* have been linked to this condition.

LEOPARD syndrome
A genetic disorder, the mnemonic name of which stands for lentigines (skin lesions), electrocardiographic conduction abnormalities (heart malfunction), ocular hypertelorism, pulmonary stenosis, abnormal genitalia, retarded growth and deafness. It is also sometimes referred to as Noonan syndrome with multiple lentigines. Mutations in *PTPN11*, *BRAF*, *CRAF* and *MEK1* have been associated with this disease.

Cardio-facio-cutaneous syndrome
A genetic disorder, the clinical presentation of which includes distinctive facial appearance, unusually sparse, brittle, curly scalp hair, a range of skin abnormalities, various heart malformations, delayed growth and foot abnormalities. Mutations in *KRAS*, *BRAF*, *MEK1* and *MEK2* have been associated with this disease.

Box 1 | **Biological functions of RAF family members**

The characterization of *RAF* loss-of-function alleles in flies and worms represented the first *in vivo* studies tying RAF to cell proliferation and cell differentiation events in metazoan development[30,32]. Knockout experiments in mice then helped to delineate the role of RAF proteins in vertebrates. Briefly, systemic loss of *BRAF* or *CRAF* caused embryonic lethality during mid-gestation owing to placental abnormalities, severe developmental defects, growth retardation, reduced cell proliferation and increased cell death[210–214] (see Supplementary information S2 (table)). By contrast, *ARAF* ablation caused mild neurological and intestinal defects[215], possibly because it has a less prominent role than BRAF and CRAF in biology or because it is redundant with other RAF isoforms. The systemic double knockout of *BRAF* and *CRAF* accelerated the onset of embryonic lethality compared with single-gene disruption[210]. Tissue-targeted double ablation of *BRAF* and *CRAF* blocked RAS-induced tumorigenesis in the skin[216] and induced premature hair greying owing to loss of melanocytes[217] (see Supplementary information S2 (table)). Single knockout of kinase suppressor of RAS 1 (*KSR1*) and *KSR2* has also been carried out in mice. *KSR1*$^{-/-}$ mice were viable with no overt phenotype. However, closer inspection in primary cells revealed widespread reduction of RAS–ERK signalling and impairment of a wealth of associated downstream events, such as reduced proliferation rate, resistance to transformation by activated RAS, defects in cell cycle re-entry following DNA repair, impeded adipogenesis, and impaired T cell activation and immunological synapse formation[61,170,218–220]. *KSR2*$^{-/-}$ mice were also viable but developed a severe obesity phenotype that has been linked to reduced ERK signalling, as well as to an energy expenditure deficit and disrupted AMP-activated protein kinase (AMPK) signalling[221,222]. Consistent with this phenotype, *KSR2* mutations have recently been connected to obesity in humans[223].

Conversely, transgenic mice expressing *BRAF* or *CRAF* gain-of-function alleles confirmed the cancer-driving potential of these genes. For example, expression of BRAF in which Val600 is mutated to Glu in the melanocyte lineage of mice or zebrafish induced senescent melanocytic masses and invasive tumours displaying hallmarks of melanoma cells[224,225] (see Supplementary information S2 (table)). The ability of kinase-impaired or weakly activated RAF mutants to induce tumours[116,226] or RASopathy-associated phenotypes[195] has also been confirmed in transgenic mouse models (see Supplementary information S2 (table)).

BRAF CRD account for a fair proportion of cardio-facio-cutaneous and Noonan syndrome cases (FIG. 6a). Changes in the RBD sequence have also been observed in samples from patients with cancer (FIG. 6a) but, as with CRD mutations, their role in driving tumorigenesis is unclear.

With respect to the CR2 domain, amino acid substitutions within the inhibitory phosphorylated epitope area that is recognized by 14-3-3 proteins are another class of mutations that are likely to de-repress RAF kinase activity (FIG. 6a). Mutation of Ser259 in *CRAF* is predominant in this region, but mutations of neighbouring residues are also observed. Although they account for a small proportion of *RAF* somatic mutations in cancer, they represent the main class of *CRAF* alterations in Noonan and LEOPARD syndromes[186,187] (FIG. 6a). Analogous BRAF mutations surrounding Ser365 are found in a small proportion of samples from patients with cancer[183] but, so far, have not been detected in RASopathies. With regard to their underlying mechanism, biochemical data show that Noonan syndrome mutations in the CR2 domain impede the inhibitory association of 14-3-3 with CRAF and promote RAS binding, which leads to recruitment of CRAF to the plasma membrane and CRAF activation[194].

Inducing RAF dimerization: mutations and inhibitors. Characterization of the diverse *BRAF* oncogenic alleles encoding proteins with impeded intrinsic kinase activity has advanced our understanding of RAF activation. Several of these mutations target key structural elements of the kinase catalytic and regulatory architecture, such as the Gly-rich P-loop or the DFG motif (FIG. 6b). Kinase-impaired BRAF mutants were shown to work through endogenous CRAF[114,115]. This was later substantiated by the finding that kinase-dead BRAF and activated RAS work synergistically in promoting tumour formation by a mechanism involving BRAF–CRAF heterodimers[116]. The discovery of the side-to-side dimerization interface and the delineation of its role in RAF allosteric transactivation provided a structural explanation for this phenomenon[60] (FIG. 6c). The increased dimerization potential of kinase-impaired BRAF mutants is now widely accepted and, in sharp contrast with the Val600Glu mutation, their oncogenic and transactivation potential depends on an intact dimerization interface and on RAS activity[124,125] (FIG. 6c). Interestingly, a kinase-impaired CRAF variant from patients with Noonan syndrome also reciprocally transactivates endogenous BRAF via a dimerization-dependent mechanism[195].

Although the allosteric control of dimerization by kinase-impaired RAF alleles is a viable route to RAF activation, kinase-active mutations can also drive RAF dimerization. For example, the BRAF-Glu586Lys mutant promotes the formation of BRAF dimers[60,125] by creating an additional contact point between two protomers in the periphery of the side-to-side interface[60]. An analogous CRAF variant (Glu478Lys) was also found in samples from patients with cancer[183], suggesting that the mechanism also operates in CRAF.

Given its causal role in cancer, a diverse set of ATP-competitive inhibitors against BRAF-Val600Glu have been designed, which target this mutation through a range of binding modes[196]. Some of these — for example, the clinically approved vemurafenib (PLX4032) — showed highly encouraging clinical response against metastatic melanoma that depends on this BRAF mutant[197]. However, most patients relapse within a year of treatment.

Diverse mechanisms causing primary or acquired resistance to RAF inhibitors have been described, and a considerable proportion of these was found to involve enhanced RTK activity or reactivation of ERK signalling[198–201]. The combination of RAF and MEK inhibitors has been tested, and early results point to a significant, albeit modest, prolongation of median disease-free survival[202]. Although acquired resistance was not unexpected, an unforeseen limitation of these inhibitors is that they induce, in a dose-dependent manner, ERK signalling in wild-type RAF tissues harbouring high RAS activity[116,131,132]. The basis for this adverse effect is not completely understood, but it is possible that drug-bound RAF molecules adopt a conformation that is prone to dimerization in the presence of high RAS activity. As a result, at non-saturating drug concentrations their ability to dimerize with drug-free protomers leads to productive transactivation and hence to the paradoxical ERK signalling[116,131,132] (FIG. 6d). Although a global 'rewiring' of the RAF dimerization network also

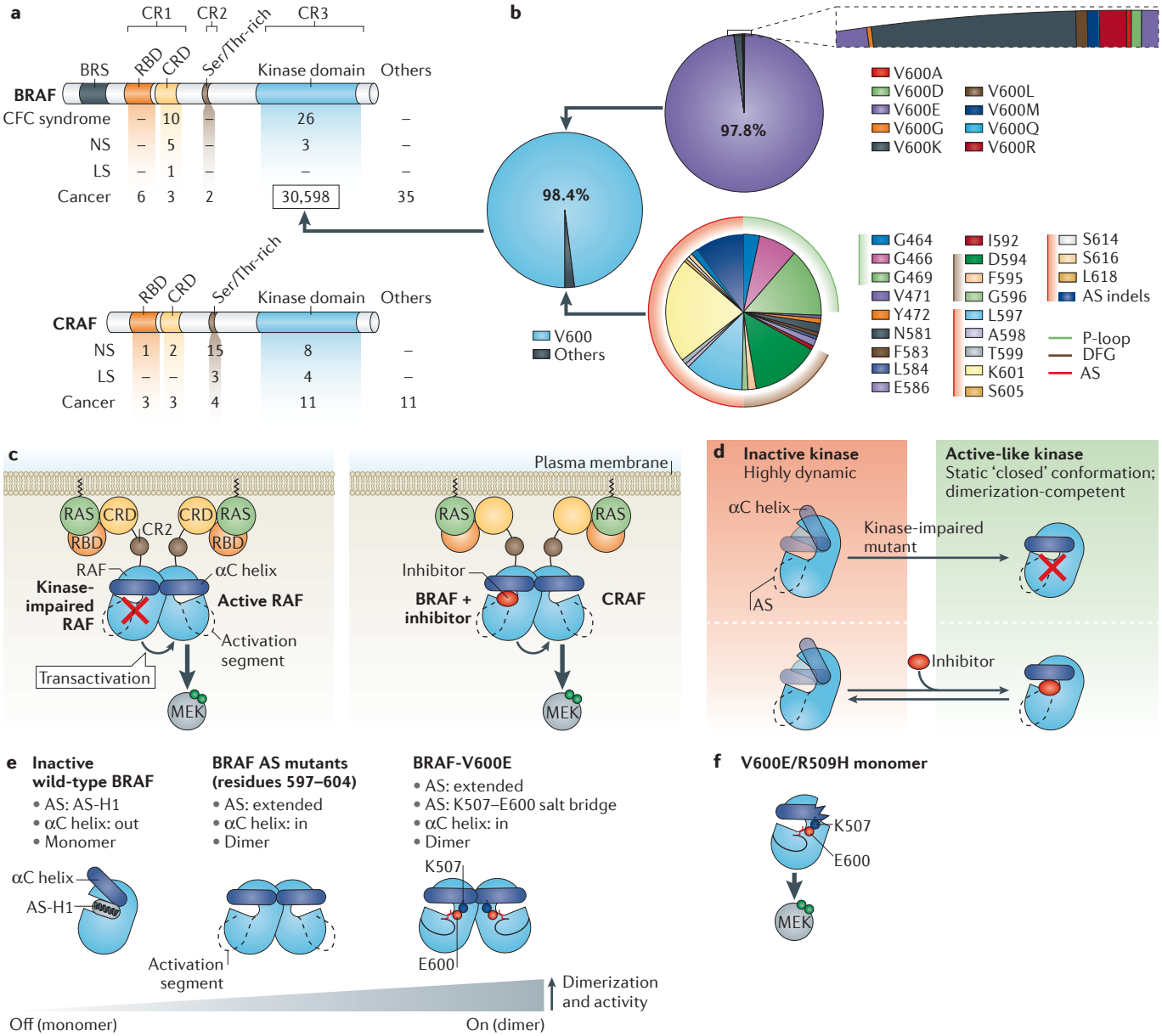


Figure 6 | **Prevalence of *RAF* mutations in human diseases.** **a** | Mutations found in *BRAF* and *CRAF* loci in somatic cancer samples (COSMIC database[183]), as well as in samples from patients with cardio-facio-cutaneous (CFC) syndrome[184,185,245,246], LEOPARD syndrome (LS)[186,245,246] and Noonan syndrome (NS)[187,245–247], have been mapped, and the numbers of mutations found in the various protein domains are shown. Most oncogenic mutations occur in the BRAF kinase domain. **b** | Pie charts depict the distribution of BRAF kinase domain mutations. V600 variants are by far the most frequently occurring lesions, accounting for ~98% of the cases. Other mutations also occur within the P-loop and DFG motif, as well as in other positions within the activation segment (AS). **c** | Kinase-impaired BRAF or CRAF show increased RAS-dependent kinase domain dimerization and thereby enhanced transactivation of other wild-type RAF protomers (left). Similarly, RAF ATP-competitive inhibitors induce ERK signalling by stimulating RAS-dependent RAF dimerization (right). **d** | Kinase-impaired RAF mutants (top) and RAF inhibitors (bottom) stimulate dimerization by restricting kinase domain dynamics. **e** | In quiescent cells, wild-type RAF is maintained in a monomeric and inactive state by the formation of AS-H1, an inhibitory helix within the AS, which precludes the inward movement of the αC helix and the attainment of the active state (left). Mutations or indels within the BRAF AS disrupt the inhibitory arrangement of AS-H1, favouring the αC helix 'in' position, dimerization and an overall active state (middle). The V600E mutation also creates a salt bridge between E600 and K507 that further stabilizes the active conformation of the AS and the dimeric active state (right). **f** | In contrast to wild-type RAF, BRAF-V600E can also act as a monomer (exemplified here by the V600E/R509H double mutant) by preserving an active conformation of the AS through the E600–K507 interaction. BRS, BRAF-specific region; CR, conserved region; CRD, Cys-rich domain; RBD, RAS-binding domain.

occurs upon inhibitor treatment, including the induction of BRAF–KSR1 heterodimers[174], spurious ERK activation seems to be primarily caused by RAS-dependent induction of CRAF-containing dimers[116,131,132].

Dimerization-driven RAF activation by kinase inhibitors or kinase-impaired RAF variants are exciting examples of the concept of allosteric regulation[203]. Mutations or compounds that bind to the kinase catalytic cleft

operate from a distance to control the side-to-side interface. The structural mechanism underlying these effects is poorly understood, but one hypothesis suggests that inhibitors (and possibly some mutations in the oncogenic kinase cleft) globally reduce BRAF kinase domain dynamics, which mostly refers to the relative mobility of the N- and C-lobes (FIG. 4b), and impose an overall closed and active-like conformation to the kinase domain lobes. This allows the kinase domain to present a static outer surface that is permissive to dimerization[121] (FIG. 6d). Consistent with this model, RAF inhibitors promote the dissociation of HSP90–CDC37 chaperone complexes from RAF, which are thought to be crucial for its proper folding[204].

Interestingly, although RAF inhibitors stimulate dimerization by stabilizing the kinase domain in a closed and rigid state, the binding of ATP derivatives reduces RAF dimerization, which suggests that ATP derivatives and RAF inhibitors oppositely augment the dynamics and flexibility of the kinase domain[121]. A better understanding of this phenomenon might lead to the development of superior RAF inhibitors that do not promote dimerization. Indeed, current efforts have led to the first generation of 'paradox-breaking' RAF inhibitors[205,206], but these require further characterization.

Finally, an alternative scenario that is not mutually exclusive with the 'closed and rigid' kinase domain might explain the paradoxical effect of RAF kinase inhibitors. In this model, ATP-competitive inhibitors are proposed to shut down an inhibitory autophosphorylation event occurring on the RAF P-loop and thereby maintain RAF activity[207]. The underlying mechanism remains unclear but nonetheless points to the fact that the action of different parallel mechanisms might contribute to the undesired side effects of current RAF-targeted therapies.

Mimicking phosphorylation of the activation segment. *BRAF*^{Val600Glu} is the most recurrent oncogenic *BRAF* allele, accounting for 97.86% of all *BRAF* mutations listed in the COSMIC database[183] (FIG. 6b). Its causal role in cancer is widely recognized and has been confirmed by several studies in diverse animal models (BOX 1). The Val600Glu mutation is located in the BRAF activation segment between the two regulatory phosphorylation sites (Thr599 and Ser602)[152] (FIG. 5c) and is therefore thought to act as a phosphomimetic substitution.

Based on modelling and crystallographic evidence, Glu600 is proposed to form a salt bridge with a basic residue (Lys507) situated in the RKTR motif of the αC helix, which constitutes the core of the side-to-side dimerization interface. This interaction was proposed to force the activation segment of BRAF-Val600Glu to fold backwards in an active conformation[114,208] (FIG. 6e). It is thus tempting to suggest that this Glu600–Lys507 salt interaction mimics some of the conformational reorganizations taking place upon dimerization, which in turn could explain why the activity of BRAF-Val600Glu does not depend on dimerization[122–124] (FIG. 6e). Consistent with this, disrupting this interaction with a Lys507Glu/Val600Glu double mutation reduces BRAF kinase activity, which becomes dependent on dimerization[176]. Whether phosphorylation in the activation segment of wild-type RAF achieves a conformational change similar to that observed in BRAF-Val600Glu remains to be established.

Less frequent mutations are also found within the BRAF activation segment (for example, basic amino acid substitutions such as Val600Lys and indels) (FIG. 6b). These mutants could not form the Glu600–Lys507 salt bridge exploited by BRAF-Val600Glu; although they are less active than the Val600Glu variant, they have considerable kinase activity[114]. A mechanism underlying these diverse molecular lesions was recently suggested by the structure of monomeric BRAF. Interestingly, BRAF mutations almost invariably fall in the AS-H1 helix, which stabilizes RAF in its inactive monomeric form[122] (FIG. 6e) (see above). Most BRAF mutations are thus thought to act, at least in part, by disrupting this inactive conformation of the activation segment. In addition, hydrophobic stacking with the αC helix seems to contribute to the increased activity of these mutants[122,209]. It will be interesting to determine whether they depend on dimerization or exploit alternative means to reach the active state.

Conclusion and perspectives

We have come a long way since RAF proteins were discovered more than 30 years ago. We can now discern the full spectrum of their isoform diversity, tissue distribution and biological functions in various species. We also have a fair idea of their role in RAS–ERK signalling as well as an extensive knowledge of the main signalling inputs and factors modulating their activity. Their relevance to cancer has been demonstrated, and potent small-molecule inhibitors, although not perfect, have emerged and have given us hope that taming unrestrained RAF activity could have positive consequences on human health. Finally, structural information on RAF family members is accumulating at an unprecedented pace. This is not only unravelling the inner workings of catalytic switching but also likely to guide us out of the shortcomings observed with current RAF inhibitors.

The complexity of RAF regulation and the importance that this class of enzymes represents for human health have sustained the interest of a large community of scientists over the years. Given the challenges and potentially the huge benefits lying ahead of us, we foresee exciting research for many years to come. With respect to unresolved issues, we note three areas where continued efforts should particularly pay off. The first one relates to a greater structural understanding of RAF proteins. In particular, solving at the atomic level the interplay between the N-terminal region and the kinase domain, as well as their regulation by RAS and 14-3-3 proteins, represents the next frontiers in this area. Second, the mechanism underlying the recruitment of RAF proteins to the plasma membrane is far from being solved. The precise sequence of events, the topology of RAF complexes anchored at the membrane, and the identity of the involved cofactors and lipids are still, for the most part, a mystery that could get remarkable answers from the use of single-molecule approaches.

COSMIC database
(Catalogue of somatic mutations in cancer database). A publicly available sequence repository and associated information that reports somatically acquired mutations found in diverse human cancers.

Indels
Insertion or deletion of nucleotides within genes.

Finally, the dimerization-mediated transactivation of RAF kinase domains and the isoform-specific asymmetry of the event have considerably changed the way we see RAF activation. Given that mammalian cells express three RAF and two KSR isoforms, which can engage each other, it will become important to address, on a systems level, their possible co-dependency and co-regulation in normal and pathophysiological conditions.

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Competing interests statement
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