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Authors

Hu, Jiancheng
Stites, Edward C
Yu, Haiyang
et al.

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Allosteric Activation of Functionally Asymmetric RAF Kinase Dimers

Jiancheng Hu[1,2], **Edward C. Stites**[1], **Haiyang Yu**[1], **Elizabeth Germino**[1], **Hiruy S. Meharena**[3,4], **Philip J. S. Stork**[5], **Alexandr P. Kornev**[3,4,5], **Susan S. Taylor**[3,4,5], and **Andrey S. Shaw**[1,2,6]

[1]Department of Pathology and Immunology, Washington University School of Medicine, 660 South Euclid, Box 8118, St. Louis, MO 63110

[2]Howard Hughes Medical Institute, Washington University School of Medicine, 660 South Euclid, Box 8118, St. Louis, MO 63110

[3]Department of Chemistry/Biochemistry, University of California San Diego, 9500 Gilman Drive, Mail code 0654, La Jolla, CA 92093

[4]Department of Pharmacology, University of California San Diego, 9500 Gilman Drive, Mail code 0654, La Jolla, CA 92093

[5]Howard Hughes Medical Institute, University of California San Diego, 9500 Gilman Drive, Mail code 0654, La Jolla, CA 92093

[5]Vollum Institute, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239

Summary

Although RAF kinases are critical for controlling cell growth, their mechanism of activation is incompletely understood. Recently, dimerization was shown to be important for activation. Here we show that the dimer is functionally asymmetric with one kinase functioning as an activator to stimulate activity of the partner, receiver kinase. The activator kinase did not require kinase activity, but did require N-terminal phosphorylation that functioned allosterically to induce *cis*-autophosphorylation of the receiver kinase. Based on modeling of the hydrophobic spine assembly, we also engineered a constitutively active mutant that was independent of Ras, dimerization, and activation loop phosphorylation. Since N-terminal phosphorylation of BRAF is constitutive, BRAF initially functions to activate CRAF. N-terminal phosphorylation of CRAF was dependent on MEK suggesting a feedback mechanism and explaining a key difference between BRAF and CRAF. Our work illuminates distinct steps in RAF activation that function to assemble the active conformation of the RAF kinase.

Introduction

The RAS-RAF-MEK-ERK signaling pathway is key for a variety of cellular functions including controlling cell proliferation and survival (Baccarini, 2005; Wellbrock et al.,

[6]Contact: ashaw@wustl.edu, staylor@ucsd.edu.

2004). Dysregulation of this pathway is also important in cancer with most tumors exhibiting mutations in RAS and/or RAF (Brose et al., 2002). While heavily studied over the last 20 years, the exact mechanism of RAF activation remains complex and largely unknown. Activation of RAF involves both phosphorylation and dephosphorylation, as well as binding to accessory proteins and lipids.

There are three isoforms of RAF: A-RAF, B-RAF and C-RAF. Each appears to have a distinct mechanism of activation (Baljuls et al., 2007; Galabova-Kovacs et al., 2006; Wimmer and Baccarini, 2010). BRAF is considered to be more active and have a simpler mechanism of activation in comparison to CRAF and ARAF (Chong et al., 2001; Rebocho and Marais, 2012; Zhang and Guan, 2000). This difference can potentially explain why BRAF mutations are so common in cancer, in contrast to CRAF and ARAF, where mutations are rarely found (Davies et al., 2002).

Recently, new insights into the mechanism of RAF activation were unexpectedly revealed by the effect of RAF inhibitors (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010). These drugs are effective inhibitors of RAF kinase activity, but when used at sub-saturating doses, they paradoxically activate the RAF pathway. Current models suggest that when RAF is bound to an inhibitor, it dimerizes with other RAF molecules, stimulating kinase activity in the partner molecule that is not bound to the inhibitor. A dimerization-dependent mechanism of RAF activation (Farrar et al., 1996; Weber et al., 2001) is supported by work showing that kinase-dead forms of BRAF, but not CRAF, are oncogenic and can allosterically activate BRAF and CRAF (Garnett et al., 2005; Wan et al., 2004). Additional experiments suggest that BRAF can activate CRAF, but CRAF cannot activate BRAF (Garnett et al., 2005; Poulikakos et al., 2010). Dimerization is, therefore, not sufficient by itself to explain the mechanism of RAF activation.

The activation of RAF requires phosphorylation at two different sites. One of these sites is the activation loop of the kinase where phosphorylation of two residues are required (Chong et al., 2001; Zhang and Guan, 2001). The other site is in a region close to the N-terminus of the kinase domain, a segment sometimes referred to as the N-terminal acidic (NtA) motif (Diaz et al., 1997; Marais et al., 1995; Mason et al., 1999). In BRAF, the NtA motif contains residues SSDD (S=serine, D=aspartic acid). Because one or more of the serines are constitutively phosphorylated, at least three of the four residues are acidic in the resting kinase (Mason et al., 1999). In CRAF and ARAF the sequence is SSYY and SGYY respectively (Y=tyrosine, G=glycine). Phosphorylation of these residues is not present in the resting kinase and is thought to occur when CRAF and ARAF are recruited to the plasma membrane by activated RAS (Morrison and Cutler, 1997). The current model suggests that at the plasma membrane, the tyrosines are phosphorylated by Src family tyrosine kinases, while the serines are phosphorylated by PAK or PKC family kinases (Fabian et al., 1993; King et al., 1998; Kolch et al., 1993). The role of PAK kinases in RAF phosphorylation is, however, controversial (Chiloeches et al., 2001).

Previously, we reported that substitution of a conserved alanine with phenylalanine in the kinase domains of CRAF (A373F), BRAF (A471F) and KSR1 (A587F) blocks ATP binding but allows for constitutive dimerization (Hu et al., 2011). We used the ability of these inactive mutants to generate RAF dimers where one component was active (the receiver) and the second (the activator) was not. Using this approach, we could determine what were the requirements for allosteric activation of RAF. We found that NtA motif phosphorylation, which is constitutive in BRAF, was necessary on the activator but not the receiver. This explains why kinase-inactive BRAF can transactivate CRAF but not vice versa. When the NtA is phosphorylated on CRAF or KSR1, however, both can function as allosteric activators of BRAF or CRAF. Importantly, allosteric activation induced cis-

autophosphorylation of the activation loop of the receiver molecule. NtA phosphorylation of CRAF involved MEK indicating a positive feedback mechanism that converted CRAF into an activator. These data help to explain the distinct roles of BRAF, CRAF and KSR1 in MAP kinase activation, and also provide a unifying model for RAF activation.

Results

RAF transactivation requires phosphorylation of the N-terminal acidic motif

Kinase-dead forms of BRAF occur in human cancers and are oncogenic (Garnett et al., 2005; Wan et al., 2004). By dimerizing with wild-type CRAF, kinase-dead forms of BRAF can activate MEK and subsequently ERK. Since our kinase-inactive mutants of CRAF and KSR1 constitutively dimerize, we had previously found that, in contrast to BRAF, expression of kinase-dead, dimerizing forms of CRAF and KSR1 have no effect on ERK activation (Hu et al., 2011). To determine what feature of BRAF accounts for this difference, we truncated ATP-binding deficient BRAF (A481F) by removing the N-terminal 434 or 454 residues and tested whether these truncated proteins could still induce ERK activation (Fig. 1A). The mutant lacking the first 434 residues could activate ERK, while the mutant lacking the first 454 residues could not. This suggested that the ability of kinase-dead forms of BRAF to activate ERK requires residues between 434 and 454.

Residues 446–449 of BRAF (SSDD, where S=serine and D=aspartic acid) form an acidic motif just N-terminal to the kinase domain. S446 is constitutively phosphorylated in BRAF and is important for activation as mutation of this residue attenuates BRAF kinase activity (Marais et al., 1997; Mason et al., 1999). To validate whether these residues are required for kinase-dead BRAF to stimulate ERK activation, we substituted four alanine residues for residues 446–449 (Fig. 1B). Overexpression of this BRAF mutant (BRAF-A481F/AAAA) did not stimulate ERK activation suggesting that these residues are critical for the ability of kinase-dead BRAF to stimulate ERK.

In CRAF, the sequence corresponding to 446–449 of BRAF is SSYY (where S = serine and Y = tyrosine) at position 338–341. In contrast to BRAF, these residues are not constitutively phosphorylated; rather their phosphorylation is induced during the process of CRAF activation (Chong et al., 2001; Mason et al., 1999). Phosphorylation of two of these residues (S338 and Y441) is required for CRAF activation as mutation of either residue to alanine abrogates kinase activity (Mason et al., 1999). To determine whether the absence of phosphorylation at these positions explains the inability of kinase-dead CRAF to stimulate ERK activation, we substituted DDEE (D=aspartic acid and E=glutamic acid) for residues 338–341 in kinase-dead CRAF (A373F). Overexpression of the CRAF mutant (A373F/DDEE) stimulated ERK phosphorylation when expressed in cells (Fig. 1C). Similarly, substitution of the equivalent sequence in KSR1 (552–555, YLQE) with DDEE, allowed a kinase-dead form of KSR1 (A587F/DDEE) to stimulate ERK activity in cells (Fig. 1D). Since position 555 is already a glutamic acid in KSR1 and because Y552 is the only residue in the sequence that could be potentially phosphorylated, we generated a mutant where only Y552 was replaced with aspartic acid. The single mutation functioned as efficiently as the DDEE mutant in ERK activation suggesting that if Y552 of KSR1 could be phosphorylated, it could function to activate ERK (Fig. 1D).

Since tyrosine phosphorylation of KSR1 has not been reported (Cacace et al., 1999) and because several predicted serine and threonine phosphorylation sites are located close to Y552 (T549/S550) (Blom et al., 2004), we tested whether acidic substitutions at these positions could stimulate ERK. Acidic residue substitution for T549 and S550 in kinase-dead KSR1 stimulated ERK activation in cells (Fig. 1D). This suggests that phosphorylation of serine/threonine or tyrosine residues in KSR1 could activate RAF. It also suggests that

phosphorylation beyond the four residue N-terminal acidic (NtA) motif could be important in transactivation.

To confirm that dimerization is required, dimerization-impairing forms of kinase-dead BRAF (R509H), CRAF (DDEE/R401H) and KSR1 (DDEE/R615H) (Rajakulendran et al., 2009) were tested. As expected, impairing dimerization of the BRAF (Fig. 1E), CRAF (Fig. 1F) and KSR1 (Fig. 1G) mutants strongly attenuated ERK activation. This confirms that kinase-dead forms of RAF or KSR1 can transactivate wild-type RAF molecules using a mechanism that requires dimerization and NtA residues. It also demonstrates that the RAF dimer is not functionally symmetrical.

The requirements for activator versus receiver kinase are distinct

We took advantage of the fact that kinase-dead forms of BRAF, CRAF and KSR1 could function as trans-activators to functionally separate the RAF dimer into an “activator” component (dead) and a “receiver” component (live), as proposed for other dimeric kinases (Zhang et al., 2006). To simplify the nomenclature, we will refer to kinase-dead BRAF (A481F) or the acidic mutants of kinase-dead CRAF (A373F/DDEE) and KSR1 (A587F/DDEE) as “activators”. The enzymatically-active form of BRAF or CRAF will be referred to as a “receiver”.

To determine whether the NtA motif was required on both the “activator” and the “receiver”, as is implied by current models, we tested whether a CRAF “receiver” mutant that cannot be phosphorylated on residues 338–341 (AAFF substituted for SSYY), could be activated by activator forms of BRAF and CRAF (Fig. 2A). Co-expression of the CRAF/AAFF receiver with activator forms of BRAF and CRAF stimulated ERK (Fig. 2A). Similar results were obtained when a BRAF receiver with four alanines substituted for residues 446–449 was tested (Fig. 2B). As expected, impairing dimerization also impaired transactivation (Fig. 2A and B). Since the R to H mutant doesn’t completely block dimerization, we also generated another mutant that more severely impaired dimerization and it showed even less transactivation (Fig. S1). Thus phosphorylation of the NtA motif is required only on the activator kinase and demonstrates that NtA phosphorylation is not required for CRAF kinase activity.

In vitro kinase assays confirmed that the receivers were catalytically activated. The CRAF-AAFF (Fig. 2C) or BRAF-AAAA (Fig. 2D) receiver mutants were co-expressed with activator forms of BRAF and CRAF. Immunoprecipitates prepared using antibodies to the epitope tag attached to the receiver kinases, were tested *in vitro* for kinase activity against MEK1. While the BRAF and CRAF receiver kinases had little detectable kinase activity by themselves, co-expression with BRAF and CRAF activators strongly induced their kinase activity towards MEK1 (Fig. 2C, D).

The mechanism of transactivation requires a conserved tryptophan

Multiple dimeric structures of BRAF and CRAF have been solved (Hansen et al., 2008; Hatzivassiliou et al., 2010; King et al., 2006; Tsai et al., 2008; Wan et al., 2004). These structures do not reveal a clear localization of the NtA motif in the dimer interface. Some of the structures of CRAF do show that a conserved tryptophan (CRAF-342, BRAF-450, KSR1-556) is a component of the dimer interface (Hatzivassiliou et al., 2010) and is positioned adjacent to the critical arginine residue required for dimerization (CRAF-R401, BRAF-R509, KSR-R615) (Fig. 3A) (Rajakulendran et al., 2009). As the conserved tryptophan is the first residue following the NtA motif (Fig. 3B), it may be important in the mechanism of transactivation. Sequence alignment showed that this tryptophan is identical

in position to W238 in LCK, W258 in SRC, and W235 in ABL, W391 in BTK and functionally equivalent to L680 of the EGF receptor (Zhang et al., 2006) (Fig. 3B).

Replacing tryptophan with alanine impaired the ability of BRAF or CRAF to function either as an activator (Fig. 3C, D) or as a receiver (Fig. 3E). Since the tryptophan is part of the dimer interface, we also tested the CRAF W342A mutant in a dimerization assay. Compared to the CRAF R401H mutant, the CRAF W342A mutant had a smaller effect on dimerization when mutated in either the activator or the receiver as measured by co-immunoprecipitation (Fig. 3F, G) or by luciferase complementation (Fig. S1). This suggests that the conserved tryptophan residue is important in positioning the NtA residues in the dimer interface for transactivation.

Dimerization induces phosphorylation of the activation loop

Since dimerization is not sufficient by itself to activate CRAF or BRAF, we considered that the NtA motif functions to induce activation loop (AL) phosphorylation, which is also required for activation (Chong et al., 2001; Zhang and Guan, 2000). A phospho-specific antibody to the AL showed that phosphorylation was induced on the receiver but not the activator kinase (Fig. 4A, compare lanes 1 versus 3). This was specific as a mutant with the AL phosphorylation sites mutated to alanine (TASA) did not react with the antibody (Fig. 4A, lanes 2 and 4). Since only the activator is not catalytically active, AL phosphorylation likely occurs by cis-autophosphorylation.

A mutation that preassembles the active conformation circumvents all of the activation steps

Cis-autophosphorylation is unusual and implies that the active conformation was formed prior to autophosphorylation. This suggested that transactivation functions to assemble the active conformation of the receiver as a prerequisite for AL phosphorylation. Previously, our work defining the hydrophobic core of active kinases proposed that all active kinases assemble a structure called the regulatory hydrophobic spine (R-Spine) (Hu et al., 2011; Taylor and Kornev, 2010). In the dimeric CRAF and BRAF structures, the R-spine is only partially assembled with interactions seen between the top two residues, BRAF: F516 and L505 (Fig. 4C, left panel) or CRAF: F408 and L397 (data not shown). The phenylalanine in the key DFG motif can be displaced rearward in the DFG “out” position in the inactive kinase (Fig. 4C, middle panel-inactive) or in the active position in the R-spine, in the DFG “in” position (Fig. 4C, left panel-active) (Hansen et al., 2008; Wan et al., 2004). We therefore considered that dimerization and the NtA residues function by positioning the DFG motif into the R-spine of the receiver, as required for the active conformation.

To test this hypothesis, we reasoned that if the R-Spine were already assembled, dimerization and the AL would be unnecessary. Modeling suggested that substitution of L397 in the αC helix with phenylalanine, a larger more hydrophobic residue, might stabilize the R-spine by promoting hydrophobic interactions with F408 above, and F487 below (Fig. 4C, right panel).

As predicted, replacement of CRAF L397 and BRAF L505 with phenylalanine resulted in a strong, constitutively active kinase consistent with assembly of the R-spine (Fig. 4B). Replacement of the two AL phosphorylation sites, T491 and S494, with alanines (TASA) of CRAF had no effect on its activity suggesting that if the R-spine is stably assembled, AL phosphorylation is no longer required (Fig. 4B). Impairing the ability of CRAF L397F or BRAF L505F to dimerize had only a small effect on either constructs’ ability to active ERK. This suggested that the R-spine mutation stabilizes the active conformation but does not do so completely. These data suggest a temporal series of events where dimerization and NtA

motif phosphorylation of the activator induces formation of the R-spine on the receiver. Assembly of the R-spine then allows for AL phosphorylation that may function to further stabilize the active conformation.

Lastly, we tested whether the CRAF L397F mutant displayed kinase activity and activation loop phosphorylation when expressed in bacteria. The CRAF L397F mutant was insoluble in bacteria, but the CRAF DDEE/L397F was both soluble, active and displayed AL phosphorylation (Fig. 4D, and data not shown). When the ATP binding deficient form of CRAF (A373F) was co-expressed with CRAF L397F in bacteria, AL phosphorylation was detected only on CRAF L397F and not CRAF A373F (Fig. 4E). Altogether, this supports a model where AL phosphorylation occurs by cis-autophosphorylation.

N-terminal acidic motif phosphorylation of CRAF is MEK dependent

Because S338 phosphorylation was not required on the CRAF receiver, we considered that S338 phosphorylation might function as a feedback mechanism that occurs after RAF activation rather than, as currently believed, before activation. Pre-treatment of cells with two different MEK inhibitors blocked a significant fraction S338 phosphorylation induced by EGF (Fig. 5A, 5B and S2) or by BRAF inhibitor (Fig. S2). This was specific as PAK and CAMKII inhibitors had no effect. This demonstrates that MEK plays a major role in the induction of S338 phosphorylation but the residual phosphorylation suggests that other mechanisms of S338 phosphorylation also exist. Co-expression of a constitutively active form of MEK with CRAF induced strong S338 phosphorylation that was completely blocked by MEK inhibition but not by dominant negative RAS further supporting a role of MEK in S338 phosphorylation (Fig. 5C, 5D). These data showing that CRAF S338 phosphorylation can occur as a consequence of CRAF activation, suggests that it functions as a positive feedback mechanism allowing the activation of CRAF to convert the CRAF receiver into a CRAF activator (depicted in Fig. 6).

Discussion

The current model of RAF activation is complex and involves dimerization, phosphorylation and dephosphorylation as well as interactions with various accessory proteins and lipids (Morrison and Cutler, 1997). How these various events are coordinated to control activation is not known. In the current model, recruitment of CRAF to the membrane allows the CRAF NtA motif to be phosphorylated by Src (Fabian et al., 1993; Marais et al., 1995), PAK and PKC kinases (King et al., 1998; Kolch et al., 1993). For BRAF, these phosphorylations are unnecessary since 446–447 are constitutively phosphorylated and 448–449 are aspartic acids (Marais et al., 1997; Mason et al., 1999). In a mechanism that was not understood, phosphorylation of the AL occurs after membrane recruitment resulting in the activation of RAF.

Here we showed that the function of the phosphorylated NtA motif is to facilitate transactivation, explaining how dimerization functions to stimulate RAF kinase activity. Our data support a model where the NtA motif engages residues in the partner kinase inducing the active conformation. This explains how BRAF inhibitor drugs can stimulate MEK and ERK activation and also why kinase inactive forms of BRAF can function as oncogenes. The state of phosphorylation of the NtA determines their ability to transactivate and is a key functional difference between the two kinases. Our work further clarifies another step in kinase activation by showing that the kinase that phosphorylates the AL is RAF itself. Lastly, our findings that NtA phosphorylation is not required for CRAF activation suggested that NtA phosphorylation could occur after, rather than before activation. Consistent with this, we found that MEK inhibition inhibits CRAF NtA phosphorylation induced by EGF.

This introduces a new complexity to the RAF activation pathway by showing that BRAF and CRAF have distinct functions in the activation of MEK.

A critical part of our approach was based on a mutant that we previously generated that is kinase-dead but can constitutively dimerize (Hu et al., 2011). This was important, as differences in dimerization efficiency might have explained differences between the ability of BRAF versus CRAF to transactivate. The use of these constitutively dimeric forms showed clearly that NtA motif phosphorylation was the major difference.

This difference between BRAF and CRAF suggests that the stoichiometry between BRAF and CRAF levels could be important. CRAF molecules are considered to be more abundant than BRAF in fibrobasts (Mikula et al., 2001). We confirmed and quantitated this showing that CRAF is about 3–4 times more abundant than BRAF in fibroblast cell lines (Fig. S3). Mathematical modeling illustrated how differences in stoichiometry between BRAF and CRAF could alter the sensitivity and magnitude of MEK activation assuming that NtA phosphorylation of CRAF occurred after activation (Fig. S4). High CRAF:BRAF ratios functioned to inhibit signals that are weak or of short duration. The reduction in signal because CRAF was initially unable to serve as an activator was minimzed as signal intensity or the duration of the signal increased. The CRAF:BRAF ratio is known to vary in tumors (Karreth et al., 2009) and we find that it can vary significantly between different cells and tissues (unpublished). Differences in activation thresholds could play an important role in determining the quality of MAP kinase activation.

Differences in expression levels between BRAF and CRAF can also explain why CRAF is considered an obligate downstream partner for BRAF. This conclusion is based on results showing that ERK activation induced by kinase-dead BRAF requires CRAF but occurs normally in the absence of BRAF (Garnett et al., 2005; Hatzivassiliou et al., 2010; Poulikakos et al., 2010; Wan et al., 2004). We confirmed these findings (Garnett et al., 2005; Wan et al., 2004) but found that, overexpressing BRAF in the CRAF deficient cell lines could rescue the ability of BRAF inhibitors to stimulate ERK (Fig. S5). This suggests that the greater abundance of CRAF expression can explain the directionality of RAF activation.

Mechanism of RAF activation

Recently we proposed a model that the assembly of two hydrophobic spines, the catalytic and the regulatory spines, can provide a unifying explanation for protein kinase activation (Kornev et al., 2008; Taylor and Kornev, 2010). We used this model to test how dimerization, NtA and AL phosphorylation can work together to facilitate RAF activation and as a strategy to generate novel mutants to confirm our hypotheses.

The regulatory spine (R-spine) is composed of four residues, all from critical sites in the kinase (Kornev et al., 2006). In BRAF, the top residue is F516 from the β4 strand of the upper lobe. The second residue is L505 from the αC helix. The third residue is F595 from the DFG sequence that defines the N-terminal side of the AL. The bottom residue is H574 from the conserved HRD motif that positions catalytic residues and the substrate for phosphate transfer. In CRAF, the four R-spine residues are F408, L397, F487 and H466. When all four residues are assembled together in linear alignment, the kinase is active.

The known function of the conserved tryptophan in SRC, HCK, CSK and ITK, suggests that dimerization may function to position the αC helix and assemble the R-spine in an active conformation (Cowan-Jacob et al., 2005; Joseph et al., 2007; LaFevre-Bernt et al., 1998; Lin et al., 2005; Xu et al., 1999). When SRC and HCK are activated, the conserved tryptophan is displaced from a hydrophobic pocket between the β4 strand and the αC helix allowing the

αC helix to swing in (Cowan-Jacob et al., 2005; LaFevre-Bernt et al., 1998). In ITK, the tryptophan is rotated in the active kinase, allowing the αC helix to move in (Joseph et al., 2007). L680 of the EGFR plays an analogous role in EGFR activation (Zhang et al., 2006). Activation of EGFR forces L680 out of the inhibitory pocket and into the dimer interface, where it plays a second role in stabilizing the dimer. It is appealing to propose a similar role for the conserved tryptophan in RAF. In RAF, the tryptophan could be playing multiple roles with distinct roles in the inhibited and active conformations as well as in the dimer interface. When a structure of inactive full length BRAF or CRAF is solved, the tryptophan may be positioned in a position that inhibits the kinase.

In the active kinase, the conserved tryptophan is in the center of the dimer interface, and the NtA residues that immediately precede the tryptophan are close by (Fig. 3A). No structures with CRAF pS338 or BRAF pS446 exist, but the known structures do show that CRAF Y340 and BRAF D448/D449 lie in the dimer interface (Hatzivassiliou et al., 2010; Qin et al., 2012; Wan et al., 2004). Without crystallographic evidence, it is impossible to know exactly how the NtA residues facilitate transactivation, but the structures show that the AL phosphorylation sites, the NtA residues and the αC helix are all situated close to the dimer interface (Baljuls et al., 2011; Wan et al., 2004) (Fig. S7).

AL phosphorylation usually thought to occur in trans by a second kinase. In the RAF dimer, AL phosphorylation appeared to occur by cis-autophosphorylation. The conundrum presented by cis-autophosphorylation is that the kinase must be primed for cis-autophosphorylation. While the exact mechanism of cis-autophosphorylation is unknown, the process of such priming for RAF must be driven by dimerization. GSK-3β, p38α and Fus3 are thought to be capable of cis-autophosphorylation and the active conformation is induced by protein binding partners (Lochhead, 2009) allowing for AL phosphorylation that then stabilizes the active conformation without the binding partner. In the case of RAF, the activator kinase must be inducing the active conformation of the receiver. Given the large number of kinases that are activated by dimerization, it is intriguing to speculate that the mechanism of cis-autophosphorylation described here might be a more general phenomenon.

We tested this model by generating a mutant with an R-spine that was constitutively assembled using the “spine” hypothesis as a model. Since the first and third residues are both phenylalanines, we predicted that substitution of a phenylalanine for the second residue, L505 (BRAF), would create a stack of three phenylalanines, stabilizing the R-spine (Fig. 4). As predicted, the L505F mutant was constitutively active and had a level of activity similar to the well-known oncogenic V600E mutant. Like the V600E mutant, the L505F mutant was also active as a monomer (Freeman et al., 2013). But the V600E mutation only works for BRAF, while the R-spine mutation activated both BRAF and CRAF supporting the broad applicability of the spine hypothesis to dissect kinase activation. AL phosphorylation also occurred when the mutated CRAF was expressed in bacteria supporting the idea that AL phosphorylation occurs by cis-autophosphorylation. That mutation of a single hydrophobic residue is sufficient to short circuit the entire regulatory pathway involving RAS, dimerization, multiple phosphorylations, and multiple binding partners illustrates the power of this mutation.

Distinct roles for CRAF, BRAF and KSR1 in ERK activation

A role for activators and receivers in RAF activation could have important implications for understanding RAF activation. When RAS is initially activated, BRAF functions mainly as an activator. If CRAF phosphorylation on 338–341 were to occur coincident with membrane recruitment and before dimerization, CRAF and BRAF would both function as activators and receivers. But if CRAF phosphorylation occurs after activation, dimers formed early in

the signaling response would be asymmetrical with one activator and one receiver kinase. Over time, as CRAF molecules become phosphorylated, they could then function as activator molecules, able to activate either BRAF or CRAF. This model (Fig. 6) is supported by our experiments showing that MEK controls NtA phosphorylation of CRAF and a recent report that MEK can activate CRAF (Leicht et al., 2013). Whether MEK can directly phosphorylate the NtA, or whether this is the property of an intermediary kinase, is not known.

Our data also have implications about the role of KSR1 in RAF activation. In its unphosphorylated state, KSR dimerization with BRAF or CRAF did not stimulate any detectable RAF kinase activity (Brennan et al., 2011; Hu et al., 2011). Interestingly, Barford and co-workers showed that BRAF dimerization with KSR2 could stimulate KSR2 kinase activity towards MEK (Brennan et al., 2011). It will be interesting to determine whether the BRAF NtA mediates this effect. By constitutively dimerizing with BRAF, unphosphorylated KSR1 could function as a buffer to block BRAF activation (McKay et al., 2011). This could be important in the resting cell, when BRAF is capable of forming functional dimers, preventing BRAF from activating itself. When KSR1 is phosphorylated, it is capable of functioning as an activator. Without better reagents, we were unable to prove that KSR1 is phosphorylated at any of the tyrosine, serine or threonine sites that we tested. But the data suggest that KSR1 phosphorylation could serve as a convergence point for upstream kinases to activate MEK and ERK in a RAS independent way. These studies reveal new ways to think about RAF isoforms and how their differences could be used to modulate signaling through the MAP kinase signaling pathway.

Methods

Biochemicals

PLX4720 (Selleck Chemicals), UO126 (Sigma), and ERK inhibitor II (Millipore) were purchased. Antibodies: anti-pERK1/2, anti-pMEK1/2, anti-pSer338 CRAF, and anti-MEK1/2 were purchased from Cell Signaling Technology; Anti-pThr598 BRAF from Thermal Scientific; anti-BRAF and anti-ERK2 from Santa Cruz Biotechnology; and HRP-labeled secondary antibodies from Jackson Laboratories.

Plasmids and Cell lines

All mutations were generated by PCR and tagged with either HA, Myc, or FLAG. pCDNA3.1 (Invitrogen) was used for transient expression and viral vectors for stable expression.

The KSR1−/− MEFs were described previously (Nguyen et al., 2002). The CRAF−/− MEFs were purchased (Huser et al., 2001). The BRAF−/− MEFs were generated in the lab.

Cell culture, Transfection, and Transduction

All cell lines were maintained in DMEM medium with 10% FBS. Cells were transfected using Lipofectamine 2000 (Invitrogen), harvested at 48 hours and lysed in RIPA buffer supplemented with protease and phosphatase inhibitors.

Stable cell lines were generated by retro- or lenti-viruses generating using 293T cells transfected respectively with appropriate viral packaging vectors. Viral supernatants were collected at 48 and 72 hours, and used to infect target cells following standard protocols (Hu et al., 2008). Transduced cells were selected using either cell sorting or antibiotics.

Immunoprecipitation, In vitro kinase assay, and Western blotting

Immunoprecipitations were performed as described before (Hu et al., 2011). Briefly, whole cell lysates were mixed with either anti-HA (Sigma), anti-FLAG (Sigma), or anti-Myc (Millipore) beads, rotated at 4°C for 60 min, and washed with RIPA buffer. For in vitro kinase assay, the immunoprecipitates were washed with kinase reaction buffer (25mM HEPES, 10mM $MgCl_2$, 0.5 mM Na_3VO_4, 0.5 mM DTT, PH7.4), and then incubated with kinase reaction buffer plus 2μg inactive MEK1 and 100μM ATP) per sample at room temperature for 30 min. Kinase reaction was stopped by adding 5μl 4 X Laemmli sample buffer. Immunoblotting was carried out as described before (Hu et al., 2011).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

Baccarini M. Second nature: biological functions of the Raf-1 “kinase”. FEBS Lett. 2005; 579:3271–3277. [PubMed: 15943972]

Baljuls A, Mahr R, Schwarzenau I, Muller T, Polzien L, Hekman M, Rapp UR. Single substitution within the RKTR motif impairs kinase activity but promotes dimerization of RAF kinase. J Biol Chem. 2011; 286:16491–16503. [PubMed: 21454547]

Baljuls A, Mueller T, Drexler HC, Hekman M, Rapp UR. Unique N-region determines low basal activity and limited inducibility of A-RAF kinase: the role of N-region in the evolutionary divergence of RAF kinase function in vertebrates. J Biol Chem. 2007; 282:26575–26590. [PubMed: 17613527]

Blom N, Sicheritz-Ponten T, Gupta R, Gammeltoft S, Brunak S. Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. Proteomics. 2004; 4:1633–1649. [PubMed: 15174133]

Brennan DF, Dar AC, Hertz NT, Chao WC, Burlingame AL, Shokat KM, Barford D. A Raf-induced allosteric transition of KSR stimulates phosphorylation of MEK. Nature. 2011; 472:366–369. [PubMed: 21441910]

Brose MS, Volpe P, Feldman M, Kumar M, Rishi I, Gerrero R, Einhorn E, Herlyn M, Minna J, Nicholson A, et al. BRAF and RAS mutations in human lung cancer and melanoma. Cancer Res. 2002; 62:6997–7000. [PubMed: 12460918]

Cacace AM, Michaud NR, Therrien M, Mathes K, Copeland T, Rubin GM, Morrison DK. Identification of constitutive and ras-inducible phosphorylation sites of KSR: implications for 14-3-3 binding, mitogen-activated protein kinase binding, and KSR overexpression. Mol Cell Biol. 1999; 19:229–240. [PubMed: 9858547]

Chiloeches A, Mason CS, Marais R. S338 phosphorylation of Raf-1 is independent of phosphatidylinositol 3-kinase and Pak3. Mol Cell Biol. 2001; 21:2423–2434. [PubMed: 11259591]

Chong H, Lee J, Guan KL. Positive and negative regulation of Raf kinase activity and function by phosphorylation. EMBO J. 2001; 20:3716–3727. [PubMed: 11447113]

Cowan-Jacob SW, Fendrich G, Manley PW, Jahnke W, Fabbro D, Liebetanz J, Meyer T. The crystal structure of a c-Src complex in an active conformation suggests possible steps in c-Src activation. Structure. 2005; 13:861–871. [PubMed: 15939018]

Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, et al. Mutations of the BRAF gene in human cancer. Nature. 2002; 417:949–954. [PubMed: 12068308]

Diaz B, Barnard D, Filson A, MacDonald S, King A, Marshall M. Phosphorylation of Raf-1 serine 338-serine 339 is an essential regulatory event for Ras-dependent activation and biological signaling. Mol Cell Biol. 1997; 17:4509–4516. [PubMed: 9234708]

Fabian JR, Daar IO, Morrison DK. Critical tyrosine residues regulate the enzymatic and biological activity of Raf-1 kinase. Mol Cell Biol. 1993; 13:7170–7179. [PubMed: 7692235]

Farrar MA, Alberol-Ila J, Perlmutter RM. Activation of the Raf-1 kinase cascade by coumermycin-induced dimerization. Nature. 1996; 383:178–181. [PubMed: 8774884]

Freeman AK, Ritt DA, Morrison DK. Effects of Raf Dimerization and Its Inhibition on Normal and Disease-Associated Raf Signaling. Mol Cell. 2013; 49:751–758. [PubMed: 23352452]

Galabova-Kovacs G, Kolbus A, Matzen D, Meissl K, Piazzolla D, Rubiolo C, Steinitz K, Baccarini M. ERK and beyond: insights from B-Raf and Raf-1 conditional knockouts. Cell Cycle. 2006; 5:1514–1518. [PubMed: 16861903]

Garnett MJ, Rana S, Paterson H, Barford D, Marais R. Wild-type and mutant B-RAF activate C-RAF through distinct mechanisms involving heterodimerization. Mol Cell. 2005; 20:963–969. [PubMed: 16364920]

Hansen JD, Grina J, Newhouse B, Welch M, Topalov G, Littman N, Callejo M, Gloor S, Martinson M, Laird E, et al. Potent and selective pyrazole-based inhibitors of B-Raf kinase. Bioorg Med Chem Lett. 2008; 18:4692–4695. [PubMed: 18676143]

Hatzivassiliou G, Song K, Yen I, Brandhuber BJ, Anderson DJ, Alvarado R, Ludlam MJ, Stokoe D, Gloor SL, Vigers G, et al. RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. Nature. 2010; 464:431–435. [PubMed: 20130576]

Heidorn SJ, Milagre C, Whittaker S, Nourry A, Niculescu-Duvas I, Dhomen N, Hussain J, Reis-Filho JS, Springer CJ, Pritchard C, et al. Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. Cell. 2010; 140:209–221. [PubMed: 20141835]

Hu J, Strauch P, Rubtsov A, Donovan EE, Pelanda R, Torres RM. Lsc activity is controlled by oligomerization and regulates integrin adhesion. Mol Imm. 2008; 45:1825–1836.

Hu J, Yu H, Kornev AP, Zhao J, Filbert EL, Taylor SS, Shaw AS. Mutation that blocks ATP binding creates a pseudokinase stabilizing the scaffolding function of kinase suppressor of Ras, CRAF and BRAF. Proc Natl Acad Sci USA. 2011; 108:6067–6072. [PubMed: 21441104]

Huser M, Luckett J, Chiloeches A, Mercer K, Iwobi M, Giblett S, Sun XM, Brown J, Marais R, Pritchard C. MEK kinase activity is not necessary for Raf-1 function. EMBO J. 2001; 20:1940–1951. [PubMed: 11296227]

Joseph RE, Min L, Andreotti AH. The linker between SH2 and kinase domains positively regulates catalysis of the Tec family kinases. Biochem. 2007; 46:5455–5462. [PubMed: 17425330]

Karreth FA, DeNicola GM, Winter SP, Tuveson DA. C-Raf inhibits MAPK activation and transformation by B-Raf(V600E). Mol Cell. 2009; 36:477–486. [PubMed: 19917255]

King AJ, Patrick DR, Batorsky RS, Ho ML, Do HT, Zhang SY, Kumar R, Rusnak DW, Takle AK, Wilson DM, et al. Demonstration of a genetic therapeutic index for tumors expressing oncogenic BRAF by the kinase inhibitor SB-590885. Cancer Res. 2006; 66:11100–11105. [PubMed: 17145850]

King AJ, Sun H, Diaz B, Barnard D, Miao W, Bagrodia S, Marshall MS. The protein kinase Pak3 positively regulates Raf-1 activity through phosphorylation of serine 338. Nature. 1998; 396:180–183. [PubMed: 9823899]

Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marme D, Rapp UR. Protein kinase C alpha activates RAF-1 by direct phosphorylation. Nature. 1993; 364:249–252. [PubMed: 8321321]

Kornev AP, Taylor SS, Ten Eyck LF. A helix scaffold for the assembly of active protein kinases. Proc Natl Acad Sci USA. 2008; 105:14377–14382. [PubMed: 18787129]

LaFevre-Bernt M, Sicheri F, Pico A, Porter M, Kuriyan J, Miller WT. Intramolecular regulatory interactions in the Src family kinase Hck probed by mutagenesis of a conserved tryptophan residue. J Biol Chem. 1998; 273:32129–32134. [PubMed: 9822689]

Leicht DT, Balan V, Zhu J, Kaplun A, Bronisz A, Rana A, Tzivion G. MEK-1 activates C-Raf through a Ras-independent mechanism. Biochim Biophys Acta. 2013; 5:978–986.

Lin X, Ayrapetov MK, Lee S, Parang K, Sun G. Probing the communication between the regulatory and catalytic domains of a protein tyrosine kinase, Csk. Biochem. 2005; 44:1561–1567. [PubMed: 15683240]

Lochhead PA. Protein kinase activation loop autophosphorylation in cis: overcoming a Catch-22 situation. Science Sig. 2009; 2:4.

Marais R, Light Y, Paterson HF, Marshall CJ. Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. EMBO J. 1995; 14:3136–3145. [PubMed: 7542586]

Marais R, Light Y, Paterson HF, Mason CS, Marshall CJ. Differential regulation of Raf-1, A-Raf, and B-Raf by oncogenic ras and tyrosine kinases. J Biol Chem. 1997; 272:4378–4383. [PubMed: 9020159]

Mason CS, Springer CJ, Cooper RG, Superti-Furga G, Marshall CJ, Marais R. Serine and tyrosine phosphorylations cooperate in Raf-1, but not B-Raf activation. EMBO J. 1999; 18:2137–2148. [PubMed: 10205168]

McKay MM, Ritt DA, Morrison DK. RAF inhibitor-induced KSR1/B-RAF binding and its effects on ERK cascade signaling. Curr Biol. 2011; 21:563–568. [PubMed: 21458265]

Mikula M, Schreiber M, Husak Z, Kucerova L, Ruth J, Wieser R, Zatloukal K, Beug H, Wagner EF, Baccarini M. Embryonic lethality and fetal liver apoptosis in mice lacking the c-raf-1 gene. EMBO J. 2001; 20:1952–1962. [PubMed: 11296228]

Morrison DK, Cutler RE. The complexity of Raf-1 regulation. Curr Opin Cell Biol. 1997; 9:174–179. [PubMed: 9069260]

Nguyen A, Burack WR, Stock JL, Kortum R, Chaika OV, Afkarian M, Muller WJ, Murphy KM, Morrison DK, Lewis RE, et al. Kinase suppressor of Ras (KSR) is a scaffold which facilitates mitogen-activated protein kinase activation in vivo. Mol Cell Biol. 2002; 22:3035–3045. [PubMed: 11940661]

Poulikakos PI, Zhang C, Bollag G, Shokat KM, Rosen N. RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. Nature. 2010; 464:427–430. [PubMed: 20179705]

Qin J, Xie P, Ventocilla C, Zhou G, Vultur A, Chen Q, Liu Q, Herlyn M, Winkler J, Marmorstein R. Identification of a novel family of BRAF(V600E) inhibitors. J Med Chem. 2012; 55:5220–5230. [PubMed: 22537109]

Rajakulendran T, Sahmi M, Lefrancois M, Sicheri F, Therrien M. A dimerization-dependent mechanism drives RAF catalytic activation. Nature. 2009; 461:542–545. [PubMed: 19727074]

Rebocho AP, Marais R. ARAF acts as a scaffold to stabilize BRAF:CRAF heterodimers. Oncogene. 2013; 32:3207–3212. [PubMed: 22926515]

Taylor SS, Kornev AP. Protein kinases: evolution of dynamic regulatory proteins. Trends Biochem Sci. 2010; 36:65–77. [PubMed: 20971646]

Tsai J, Lee JT, Wang W, Zhang J, Cho H, Mamo S, Bremer R, Gillette S, Kong J, Haass NK, et al. Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity. Proc Natl Acad Sci U S A. 2008; 105:3041–3046. [PubMed: 18287029]

Wan PT, Garnett MJ, Roe SM, Lee S, Niculescu-Duvaz D, Good VM, Jones CM, Marshall CJ, Springer CJ, Barford D, et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell. 2004; 116:855–867. [PubMed: 15035987]

Weber CK, Slupsky JR, Kalmes HA, Rapp UR. Active Ras induces heterodimerization of cRaf and BRaf. Cancer Res. 2001; 61:3595–3598. [PubMed: 11325826]

Wellbrock C, Karasarides M, Marais R. The RAF proteins take centre stage. Nat Rev Mol Cell Biol. 2004; 5:875–885. [PubMed: 15520807]

Wimmer R, Baccarini M. Partner exchange: protein-protein interactions in the Raf pathway. Trends Biochem Sci. 2010; 35:660–668. [PubMed: 20621483]

Xu W, Doshi A, Lei M, Eck MJ, Harrison SC. Crystal structures of c-Src reveal features of its autoinhibitory mechanism. Mol Cell. 1999; 3:629–638. [PubMed: 10360179]

Zhang BH, Guan KL. Activation of B-Raf kinase requires phosphorylation of the conserved residues Thr598 and Ser601. EMBO J. 2000; 19:5429–5439. [PubMed: 11032810]

Zhang BH, Guan KL. Regulation of the Raf kinase by phosphorylation. Exp Lung Res. 2001; 27:269–295. [PubMed: 11293329]

Zhang X, Gureasko J, Shen K, Cole PA, Kuriyan J. An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. Cell. 2006; 125:1137–1149. [PubMed: 16777603]

Highlights

1. RAF dimers are asymmetric with one component allosterically activating the other.
2. N-terminal phosphorylation but not kinase activity is required on the activator.
3. Dimerization induces cis-autophosphorylate of the RAF activation loop.
4. Mutations that stabilize the active conformation do not require dimerization.

Allosteric Activation of Functionally Asymmetric RAF Kinase Dimers

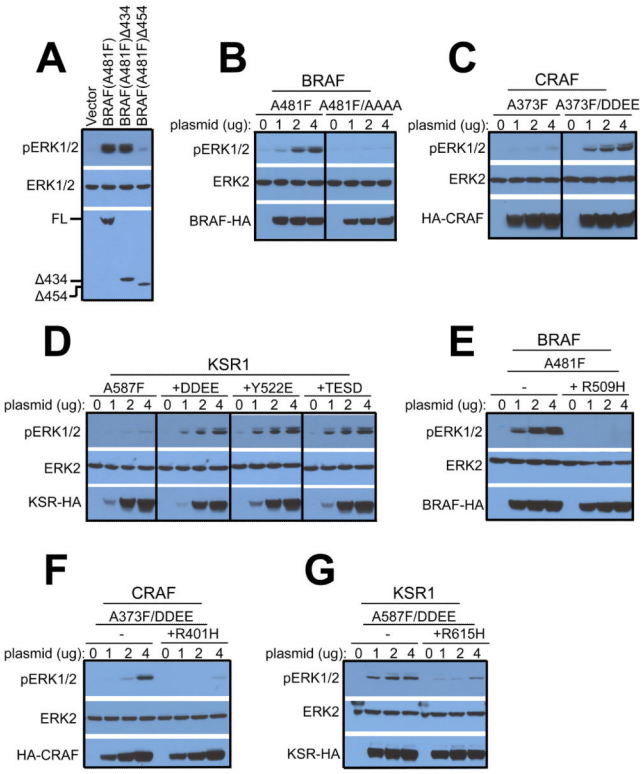

Figure 1. The ability of kinase-dead BRAF to activate ERK requires phosphorylation of the N-terminal acidic motif (NtA)

A. The NtA is required for BRAF transactivation. Kinase-dead full-length BRAF (A481F) (lane 2) or truncated forms of BRAF A481F (Δ434-lane 3, Δ454-lane 4) were transiently overexpressed in 293 cells and cell lysates immunoblotted for ERK activation (pERK) after 24 hours. **B**. Substitution of alanines (AAAA) for residues 446–449 of BRAF A481F impaired ERK activation. Expression and immunoblotting were as described in A. **C**. Substitution of acidic residues (DDEE) for residues 338–341 of kinase-dead CRAF (A373F) conferred the ability to transactivate. ERK activation was measured as described in A. **D**. Acidic residues substituted in dimerization-competent, kinase-dead KSR1 (A587F) confers the ability to transactivate. Overexpression of kinase-dead KSR1 (A587F) had no effect on ERK activation, while substitution of acidic residues (DDEE) for residues 552–555 of KSR1, for Y522 (Y522E), or for T549 and S550 (TESD) could confer the ability to transactivate. In all panels, the mutants were overexpressed as analyzed as in **A**. Transactivation by kinase-dead BRAF (**E**), CRAF (**F**) or KSR1 (**G**) involves dimerization. Replacement of an arginine residue critical for efficient dimerization impaired the ability of BRAF A481F **(E)** and mutated forms of CRAF (A383F/DDEE) **(F)** and KSR1 (A587F/DDEE) **(G)** to activate ERK. ERK activation was measured as in A.

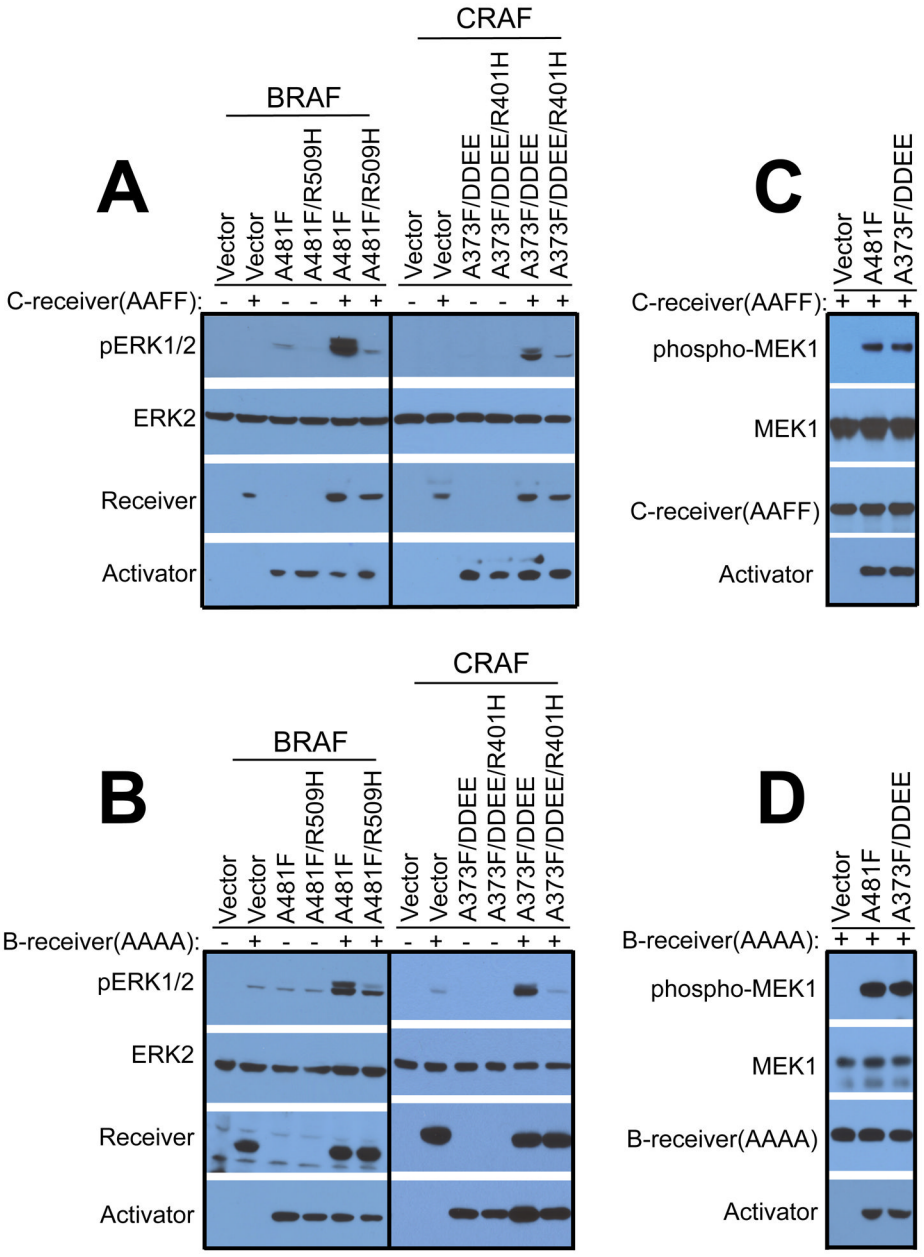

Figure 2. Phosphorylation of the acidic motif is required on the activator but not the receiver RAF kinase

A. A mutated form of truncated, catalytically active CRAF (Δ1–322) with AAFF substituted for residues 338–341 was transiently co-expressed with kinase-dead BRAF (A481F, Δ1-434 – left panel) or with acidic, kinase-dead CRAF (A373F/DDEE, Δ1–322 – right panel) with and without dimerization mutations (R509H or R401H) in 293 cells. Cell lysates were immunoblotted with antibodies to pERK1/2, ERK2, MYC (receiver constructs) or FLAG (activator constructs). **B.** A mutated form of truncated, catalytically active BRAF (Δ1–434) with AAAA substituted for residues 446–449 was transiently co-expressed with kinase-dead BRAF (A481F, Δ1–434 – left panel) or with acidic, kinase-dead CRAF (A373F/DDEE, Δ1–322 – right panel). Impairing dimerization (BRAF/R509H, CRAF/R401H) impaired transactivation. See also Figure S1. Cell lysates were analyzed as described in A. **C and D.**

Activation of receiver CRAF (C) or BRAF (D) kinase activity measured by in vitro kinase reaction. The CRAF (AAFF, Δ1–322) or BRAF (AAAA, Δ1–434) receiver constructs were co-expressed with empty vector, BRAF or CRAF activator constructs. Immunoprecipitates were prepared using an antibody to the MYC tag present on the receiver. In vitro kinase reactions were performed with purified MEK as the substrate and measured by immunoblotting with antibodies to pMEK. Immunoblotting with antibodies to MYC (receiver) or FLAG (activator) confirmed similar levels of expression.

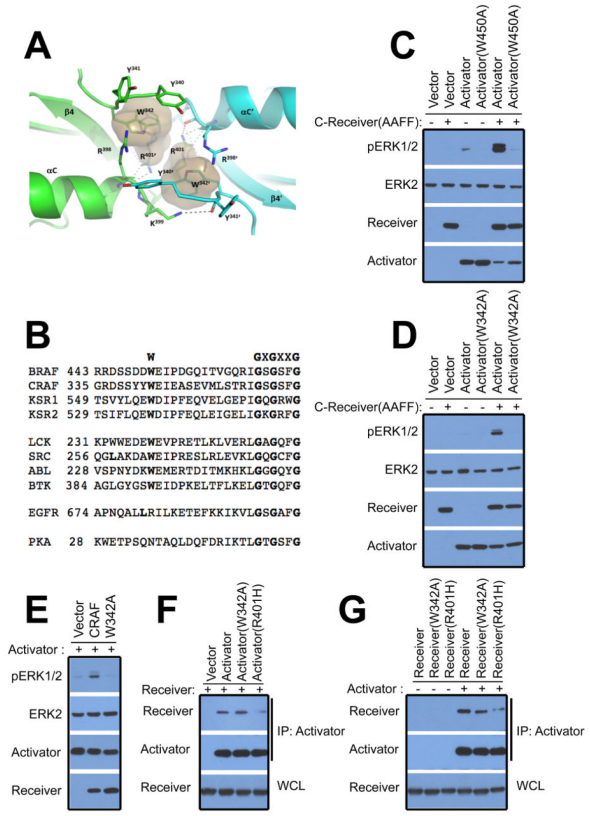

Figure 3. The mechanism of transactivation involves a conserved tryptophan

A. Schematic diagram showing the position of the W342 in the CRAF dimer interface. Note the close proximity of W342 to R401, the residue critical for dimerization and the position of the unphosphorylated Y340 and Y341. Blue and green are used to distinguish the two components of the dimer. Residue numbers between each kinase are also distinguished with a “prime”. **B.** Alignment of the N-terminal motifs of human BRAF, CRAF, KSR1, KSR2, LCK, SRC, ABL, BTK, EGFR and PKA show the conservation of the tryptophan in all of the kinases except for PKA and EGFR. In EGFR, L680 is the functional equivalent to the tryptophan (Zhang et al., 2006). **C and D**. Mutation of the tryptophan on the activator kinase impairs ERK activation. Activator forms of BRAF (**C**) or CRAF (**D**) with and without the tryptophan mutation (BRAF W450A, or CRAF W342A) were transiently co-expressed in 293 cells with CRAF receiver (AAFF, Δ1–322) in cells and cell lysates immunoblotted with antibodies to pERK, ERK2, HA (activator) and Myc (receiver). **E.** Mutation of tryptophan on the receiver impairs ERK activation. CRAF activator construct (CRAF A373F/DDEE, Δ1–322) was transiently co-expressed in 293 cells with either empty vector, CRAF receiver (AAFF, Δ1–322) or with CRAF receiver with the tryptophan mutated (AAFF/W342A, Δ1–322). Cell lysates were prepared and immunoblotted with antibodies to pERK, ERK2, HA (activator) and Myc (receiver). **F.** Mutation of the tryptophan on the activator only modestly impairs dimerization. A CRAF receiver construct (AAFF, Δ1–322) was co-expressed with vector alone, a CRAF activator (A373F/DDEE) CRAF activator with the tryptophan mutation (A373F/DDEE/W342A), or the CRAF activator with the R401H dimerization mutation. Immunoprecipitates were made with antibodies to the activator (HA) and immunoblotted with antibodies to the receiver (Myc). See also Figure S1. **G.** Mutation of the tryptophan on the receiver only moderately impairs dimerization. The CRAF receiver construct (CRAF AAFF, Δ1–322), with the tryptophan mutation (W342A) or the dimerization mutation (R401H) was transiently expressed alone or co-expressed with a

CRAF activator construct (A373F/DDEE, Δ1–322). Immunoprecipitates were made with antibodies to the activator (HA) and immunoblotted with antibodies to the receiver (Myc).

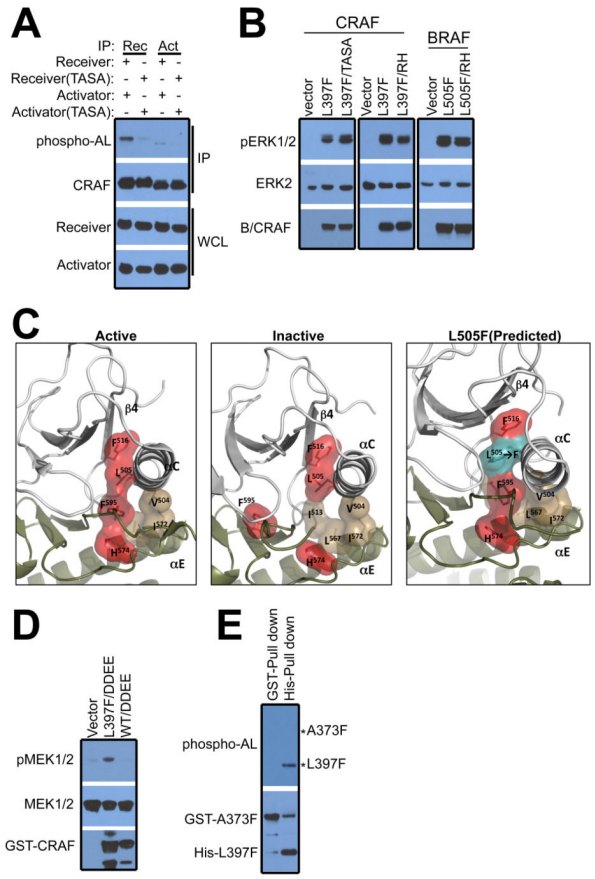

Figure 4. Constitutive assembly of the R-spine results in an active kinase that does not require dimerization or AL phosphorylation

A. Phosphorylation of the AL is induced on the receiver but not the activator. An HA-tagged CRAF activator (A373F/DDEE, Δ1–322) was co-expressed with a FLAG-tagged CRAF receiver (AAFF, Δ1–322). Immunoprecipitates were made to the receiver (anti-FLAG, lanes 1 and 2) or to the activator (anti-HA, lanes 3 and 4) and immunoblotted with a phospho-specific antibody to BRAF pT599 which also specifically reacts with pT491 of CRAF. Mutation of the AL phosphorylation sites (T491A, S494A) verified the specificity of the antibody (lanes 2 and 4). **B.** CRAF L397F and BRAF L505F mutants are constitutively active and do not require AL phosphorylation or dimerization. CRAF L397F and BRAF L505F mutants were transiently overexpressed in 293 cells and cell extracts immunoblotted with antibodies to pERK, ERK2 or to HA (CRAF or BRAF). CRAF mutant with mutations in the AL phosphorylation sites (T491A, S494A) had no effect on ERK activation. Similarly, dimerization mutants of the CRAF L397F mutant (R401H or RH) and BRAF L505F (R509H or RH) had only minor effects on ERK activation. **C.** Schematic diagram showing the positions of the R-spine residues in BRAF. In the left panel, the conformation of the R-spine in the active conformation is shown (PDB:4E26). In the middle panel, the position of the residues of the R-spine in the inactive, dimeric structure of BRAF is shown (PDB:1UWH). Note the rearward displacement of F595. In the right panel, a model predicting the structure of the L397F mutant is shown. This was modeled using TINKER (http://dasher.wustl.edu/tinker/). **D.** Kinase activity of bacterial expressed CRAF L397F/DDEE mutant. GST fusion protein of L397F/DDEE or wild-type CRAF/DDEE kinase domain was purified with GST and tested in vitro for kinase activity in vitro towards purified MEK. **E.** Co-expression of His-tagged CRAF L397F/DDEE construct with GST-

CRAF/A373F mutant in bacteria. Bacteria were lysed and proteins purified with glutathione or Ni^{++} beads and immunoblotted with BRAF pT599 antibody.

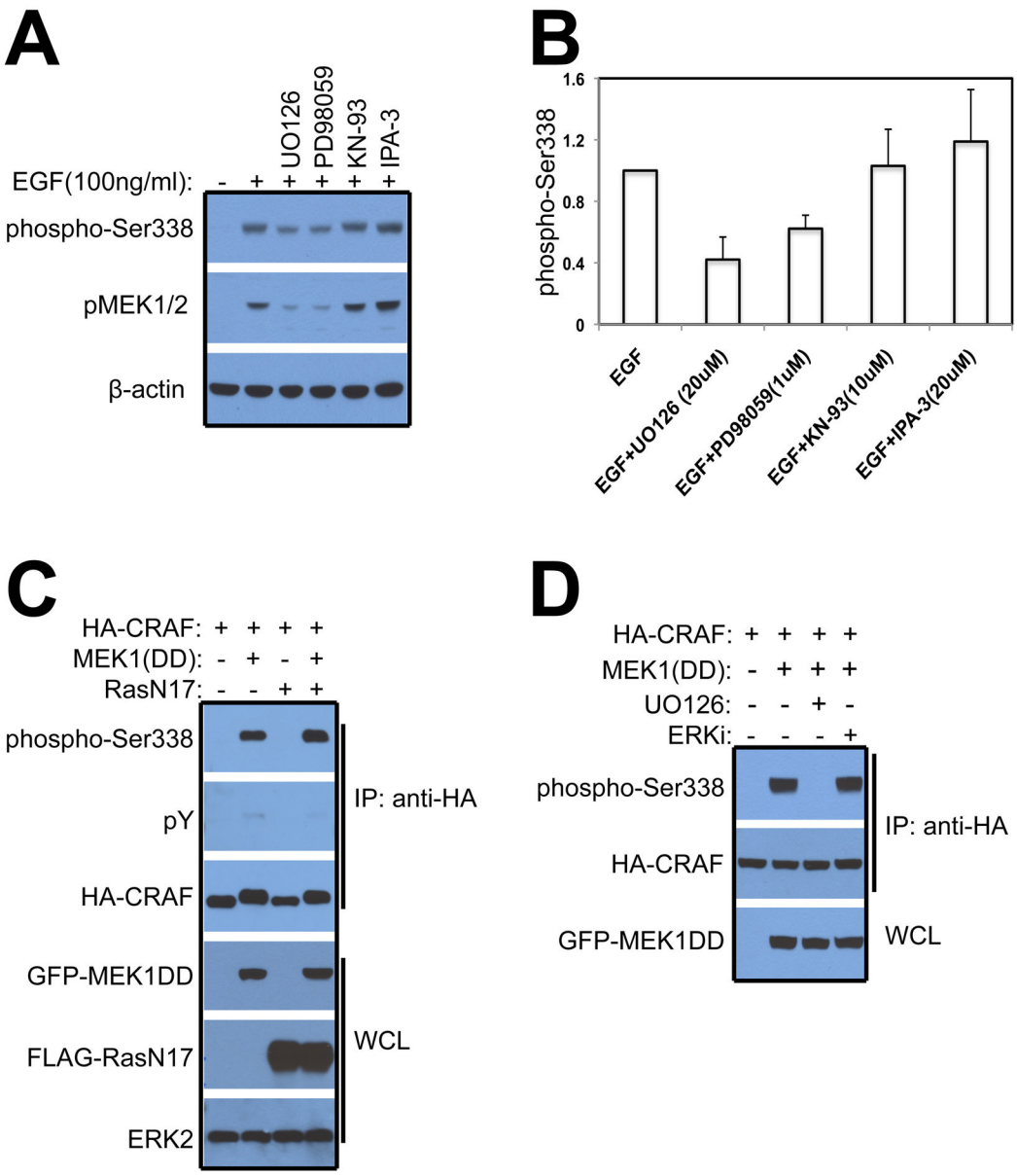

Figure 5. CRAF S338 phosphorylation is stimulated by MEK activation

A. MEK inhibitors inhibit CRAF S338 phosphorylation induced by EGF. Untransfected 293 cells were pre-treated with MEK inhibitor (UO126-20uM or PD98059-1uM), PAK inhibitor (IPA3-20uM), CAMKII inhibitor (KN-93-10uM) or vehicle for 2 hours before addition of EGF (100ng/ml). Cells were lysed after 5 minutes and CRAF S338 and MEK phosphorylation assessed using phosphor-specific antibodies. See also Figure S2. **B.** Quantification of the effect of kinase inhibitors on CRAF S338 phosphorylation. Results from 4 separate experiments are presented as mean −/+ SEM. The amount of S338 phosphorylation from the EGF treatment alone was set as 1. **C.** Co-expression of constitutively active MEK with CRAF induces S338 phosphorylation. Wild-type HA-CRAF was co-expressed with constitutively active MEK1 (DD) with and without dominant negative RAS (RasN17). Cells were lysed after 24 hours and immunoprecipitates prepared with antibodies to HA. CRAF S338 phosphorylation was assessed using a phosphor-specific antibody. **D.** MEK inhibition blocks CRAF S338 phosphorylation induced by co-expression

with constitutively active MEK. Cells were prepared as described in B, except that MEK (UO126) or ERK inhibitor (ERK inhibitor II) was added 24 hours after transfection. Cells were lysed 2 hours after addition of the inhibitor. Immunoprecipitates were analyzed as in B.

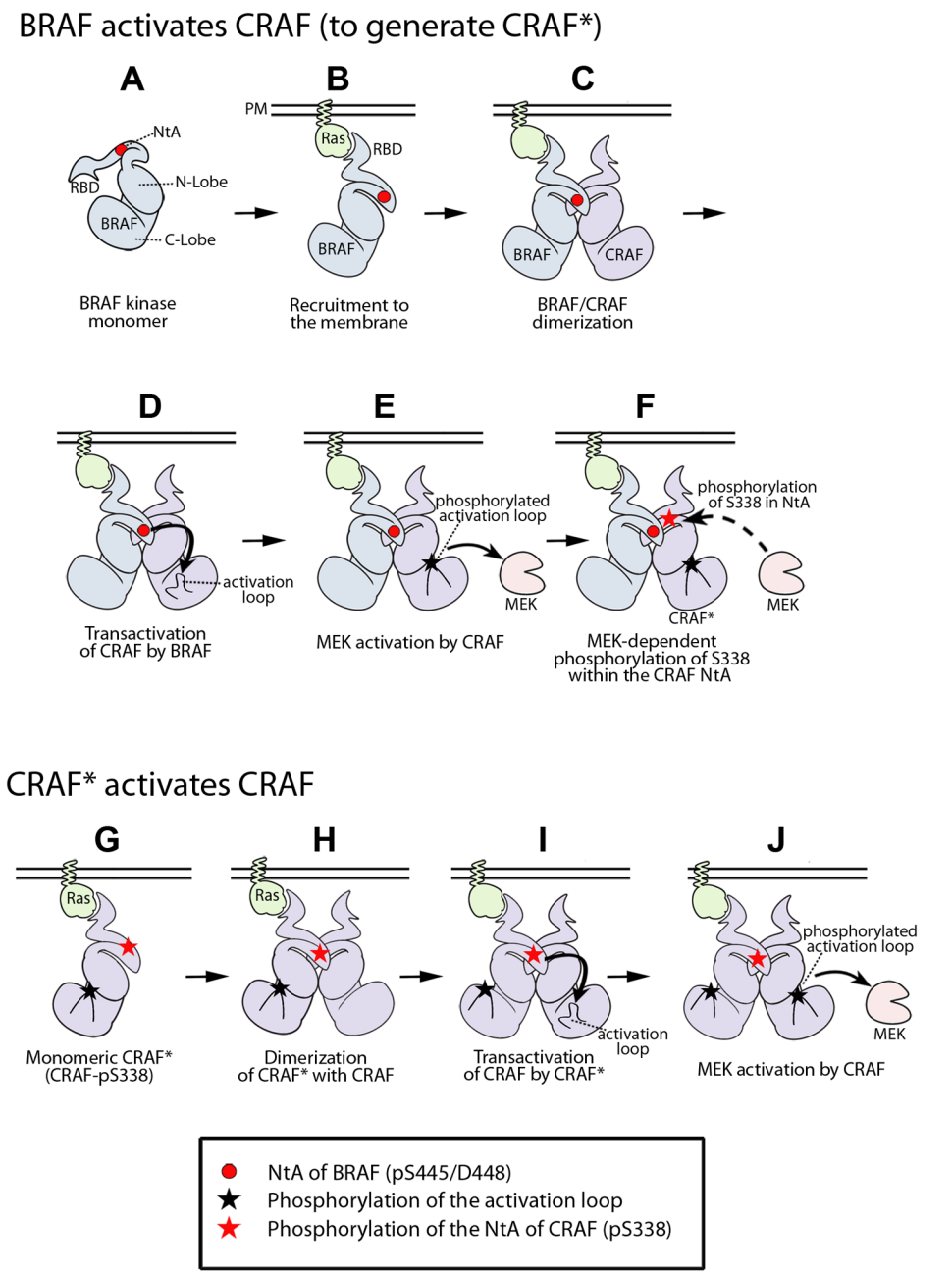

Figure 6. Model of RAF transactivation

A–F: BRAF (activator) activates CRAF (receiver). A. Domains of the RAF monomer. The two lobes of the kinase, the Ras binding domain (RBD) and the N-terminal acidic domain (NtA) are shown. BRAF is shown here with its constitutively acidic NtA (pS445/D448) depicted as a red circle. **B**. Recruitment of BRAF to the plasma membrane (PM) by GTP-RAS induces a new conformation in the N-terminal half of BRAF. **C**. RAS binding allows two RAF molecules to dimerize. A BRAF/CRAF dimer is shown here, with BRAF serving as the “activator” and CRAF as the “receiver”. **D**. For BRAF, the NtA is constitutively phosphorylated (red dot) and lies within the dimer interface. This allows it to transactivate the RAF receiver, in this case CRAF. **E**. Transactivation results in cis-autophosphorylation producing an active CRAF kinase that can then phosphorylate MEK. **F**.

The S338 of the CRAF receiver can be phosphorylated in a MEK-dependent manner. CRAF-pS338 is designated as “CRAF*”. **G-J: CRAF* activates CRAF. G**. Monomeric CRAF* dissociates from BRAF. **H**. CRAF* (the “activator”) dimerizes withCRAF (the “receiver”). CRAF * may also dimerize with, and activate, a molecule ofBRAF. **I**. The phosphorylated NtA of the CRAF* activator lies within the dimer interface and transactivates the CRAF receiver. **J.** Transactivation results in cis-autophosphorylation producing an active CRAF kinase that can then phosphorylate MEK.