

Allosteric Activation of Functionally Asymmetric RAF Kinase Dimers

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

¹Department of Pathology and Immunology

²Howard Hughes Medical Institute

Washington University School of Medicine, 660 South Euclid, Box 8118, St. Louis, MO 63110, USA

³Department of Chemistry/Biochemistry

⁴Department of Pharmacology

⁵Howard Hughes Medical Institute

University of California San Diego, 9500 Gilman Drive, Mail code 0654, La Jolla, CA 92093, USA

⁶Vollum Institute, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239, USA

*Correspondence: staylor@ucsd.edu (S.S.T.), ashaw@wustl.edu (A.S.S.)

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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. As 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., 2004). Dysregulation of this pathway is also important in cancer with most tumors exhibiting mutations in RAS and/or RAF (Brose et al., 2002). Although 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, 2013; 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 subsaturating 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 (AL) of the kinase where phosphorylation of two residues is 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 sequences are 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, whereas the serines are phosphorylated by p21-activated kinase (PAK) or protein kinase C (PKC) family kinases (Fabian et al., 1993; King et al., 1998; Kolch et al., 1993). The role of PAKs 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 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 AL of the receiver molecule. NtA phosphorylation of CRAF involved mitogen-activated protein kinase kinase (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 mitogen-activated protein kinase (MAPK) activation and also provide a unifying model for RAF activation.

RESULTS

RAF Transactivation Requires Phosphorylation of the NtA 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 extracellular signal-regulated kinase (ERK). 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 (Figure 1A). The mutant lacking the first 434 residues could activate ERK, whereas 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) 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 (Figure 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 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 (where 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 (Figure 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 (Figure 1D). Because 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 (Figure 1D).

Because 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 (Figure 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 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 (Figure 1E), CRAF (Figure 1F), and KSR1 (Figure 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 transactivators 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

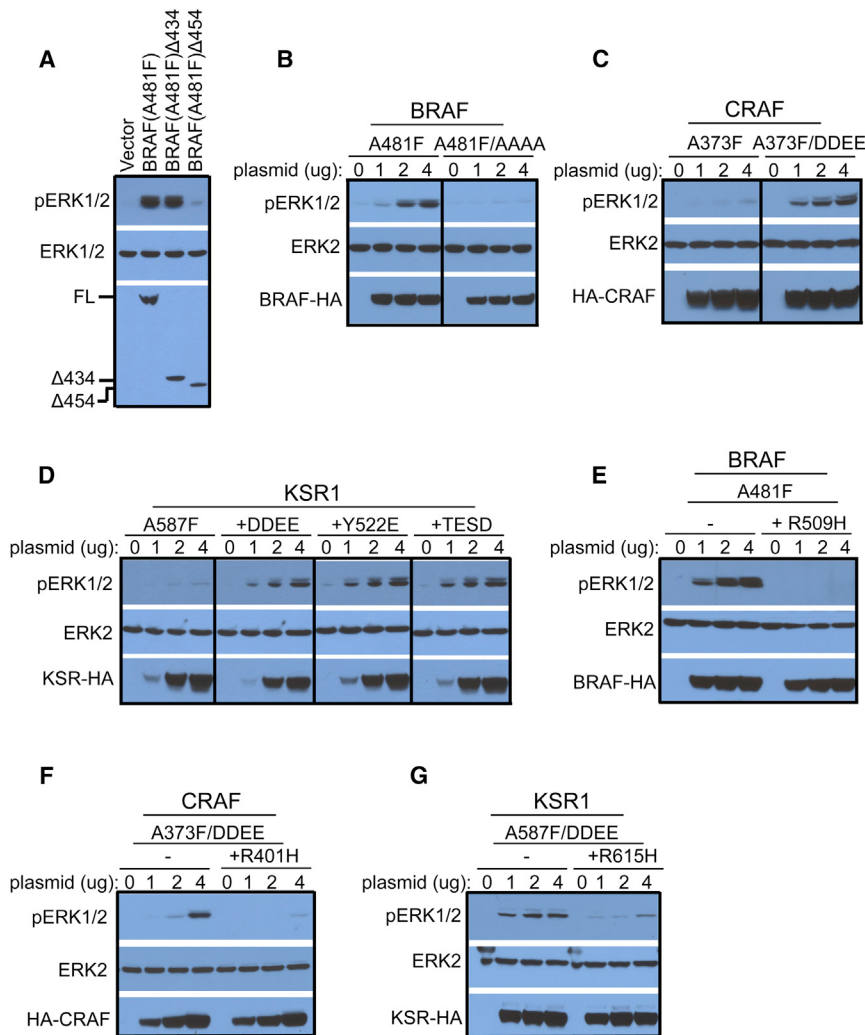


Figure 1. The Ability of Kinase-Dead BRAF to Activate ERK Requires Phosphorylation of the NtA

(A) The NtA is required for BRAF transactivation. Kinase-dead full-length BRAF (A481F) (lane 2) or truncated forms of BRAF A481F (Δ 434—lane 3, Δ 454—lane 4) were transiently overexpressed in 293 cells and cell lysates immunoblotted for ERK activation (pERK) after 24 hr.

(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) confer the ability to transactivate. Overexpression of kinase-dead KSR1 (A587F) had no effect on ERK activation, whereas 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 and analyzed as in (A). (E–G) 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).

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 (Figure 2A). Coexpression of the CRAF/AAFF receiver with activator forms of BRAF and CRAF stimulated ERK (Figure 2A). Similar results were obtained when a BRAF receiver with four alanines substituted for residues 446–449 was tested (Figure 2B). As expected, impairing dimerization also impaired transactivation (Figures 2A and 2B). Given that 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 (Figure S1 available online). 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 (Figure 2C) or BRAF-AAAA (Figure 2D) receiver mutants were coexpressed with activator forms of BRAF and CRAF. Immunoprecipitates prepared with antibodies to the epitope tag attached to the receiver kinases were tested in vitro for kinase activity against MEK1. Although the BRAF and CRAF receiver kinases had little detectable kinase activity by themselves, coexpression with BRAF and CRAF activators strongly induced their kinase activity toward MEK1 (Figures 2C and 2D).

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) (Figure 3A) (Rajakulendran et al., 2009). As the conserved tryptophan is the first residue following the NtA motif (Figure 3B), it may be important in the

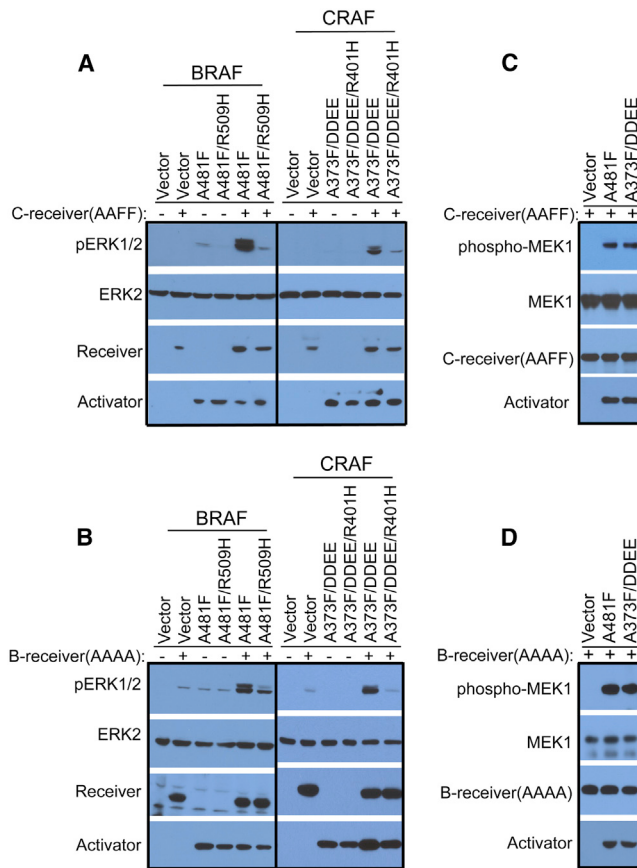


Figure 2. Phosphorylation of the Acidic Motif Is Required on the Activator but Not the Receiver RAF Kinase

(A) A mutated form of truncated, catalytically active CRAF ($\Delta 1-322$) with AAFF substituted for residues 338–341 was transiently coexpressed with kinase-dead BRAF (A481F, $\Delta 1-434$ —left panel) or with acidic, kinase-dead CRAF (A373F/DDEE, $\Delta 1-322$ —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 ($\Delta 1-434$) with AAAA substituted for residues 446–449 was transiently coexpressed with kinase-dead BRAF (A481F, $\Delta 1-434$ —left panel) or with acidic, kinase-dead CRAF (A373F/DDEE, $\Delta 1-322$ —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, $\Delta 1-322$) or BRAF (AAAA, $\Delta 1-434$) receiver constructs were coexpressed 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.

mechanism of transactivation. Sequence alignment showed that this tryptophan is identical in position to W238 in LCK, W258 in SRC, W235 in ABL, and W391 in BTK and functionally equivalent to L680 of the epidermal growth factor receptor (EGFR) (Zhang et al., 2006) (Figure 3B).

Replacing tryptophan with alanine impaired the ability of BRAF or CRAF to function either as an activator (Figures 3C and 3D) or as a receiver (Figure 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 coimmunoprecipitation (Figures 3F and 3G) or by luciferase complementation (Figure 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 AL

Since dimerization is not sufficient by itself to activate CRAF or BRAF, we considered that the NtA motif functions to induce AL phosphorylation, which is also required for activation (Chong et al., 2001; Zhang and Guan, 2000). A phosphospecific antibody to the AL showed that phosphorylation was induced on the receiver but not the activator kinase (Figure 4A, compare lane 1 versus 3). This was specific as a mutant with the AL phosphorylation sites mutated to alanine (TASA) did not react with the antibody (Figure 4A, lanes 2 and 4). Since 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) (Taylor and Kornev, 2011). 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 (Figure 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 (Figure 4C, middle panel—inactive) or in the DFG “in” position in the active position in the R-spine (Figure 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 was already assembled, dimerization and AL phosphorylation 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 (Figure 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 (Figure 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 not

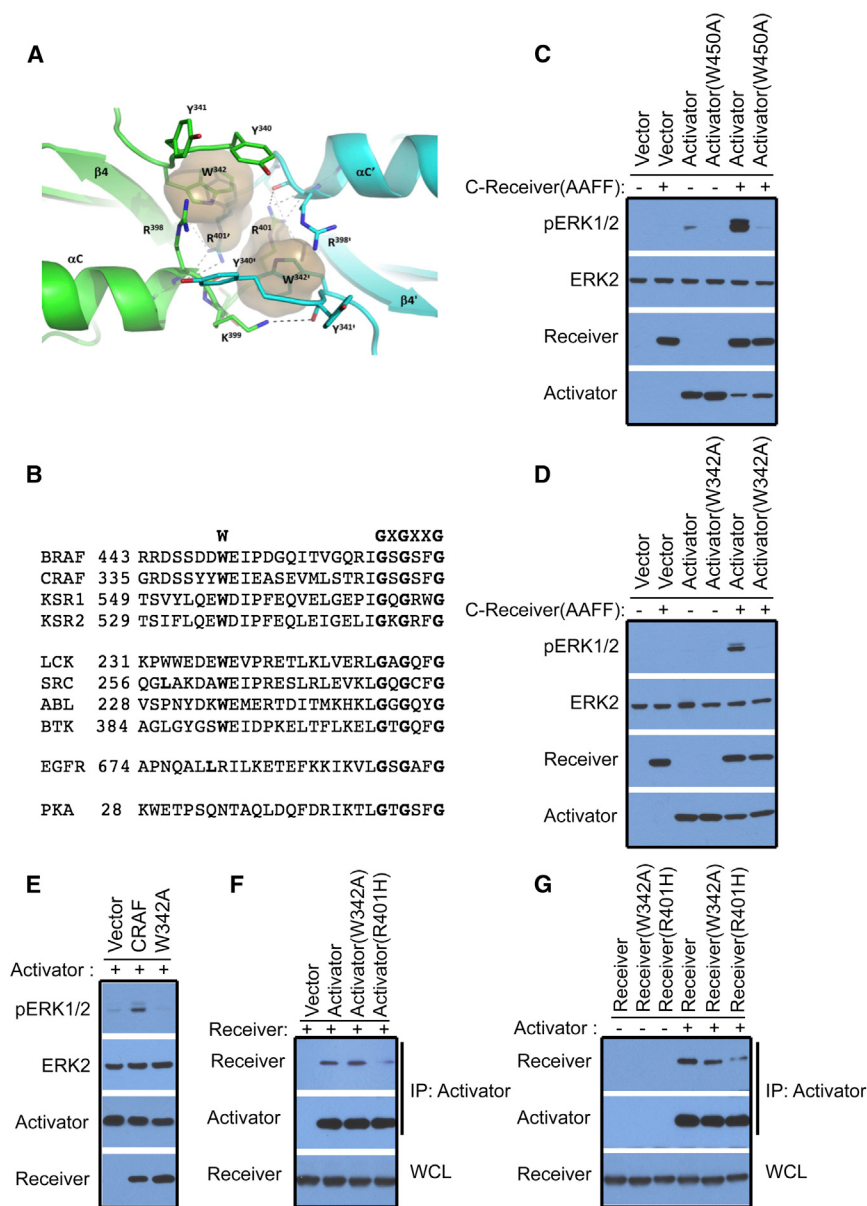


Figure 3. The Mechanism of Transactivation Involves a Conserved Tryptophan

(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 shows the conservation of the tryptophan in all of the kinases except for PKA and EGFR. In EGFR, L680 is the functional equivalent of 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 coexpressed in 293 cells with CRAF receiver (AAFF, Δ 1–322) 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, Δ 1–322) was transiently coexpressed in 293 cells with either empty vector, CRAF receiver (AAFF, Δ 1–322), or CRAF receiver with the tryptophan mutated (AAFF/W342A, Δ 1–322). 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, Δ 1–322) was coexpressed with vector alone, a CRAF activator (A373F/DDEE), the 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, Δ 1–322) with the tryptophan mutation (W342A) or the dimerization mutation (R401H) was transiently expressed alone or coexpressed with a CRAF activator construct (A373F/DDEE, Δ 1–322). Immunoprecipitates were made with antibodies to the activator (HA) and immunoblotted with antibodies to the receiver (Myc).

longer required (Figure 4B). Impairing the ability of CRAF L397F or BRAF L505F to dimerize had only a small effect on either construct's ability to activate 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 induce 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 AL phosphorylation when expressed in bacteria. The CRAF L397F mutant was insoluble in bacteria, but the

CRAF DDEE/L397F was soluble, active, and displayed AL phosphorylation (Figure 4D and data not shown). When the ATP-binding-deficient form of CRAF (A373F) was coexpressed with CRAF L397F in bacteria, AL phosphorylation was detected only on CRAF L397F and not CRAF A373F (Figure 4E). Altogether, this supports a model where AL phosphorylation occurs by *cis*-autophosphorylation.

NtA 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

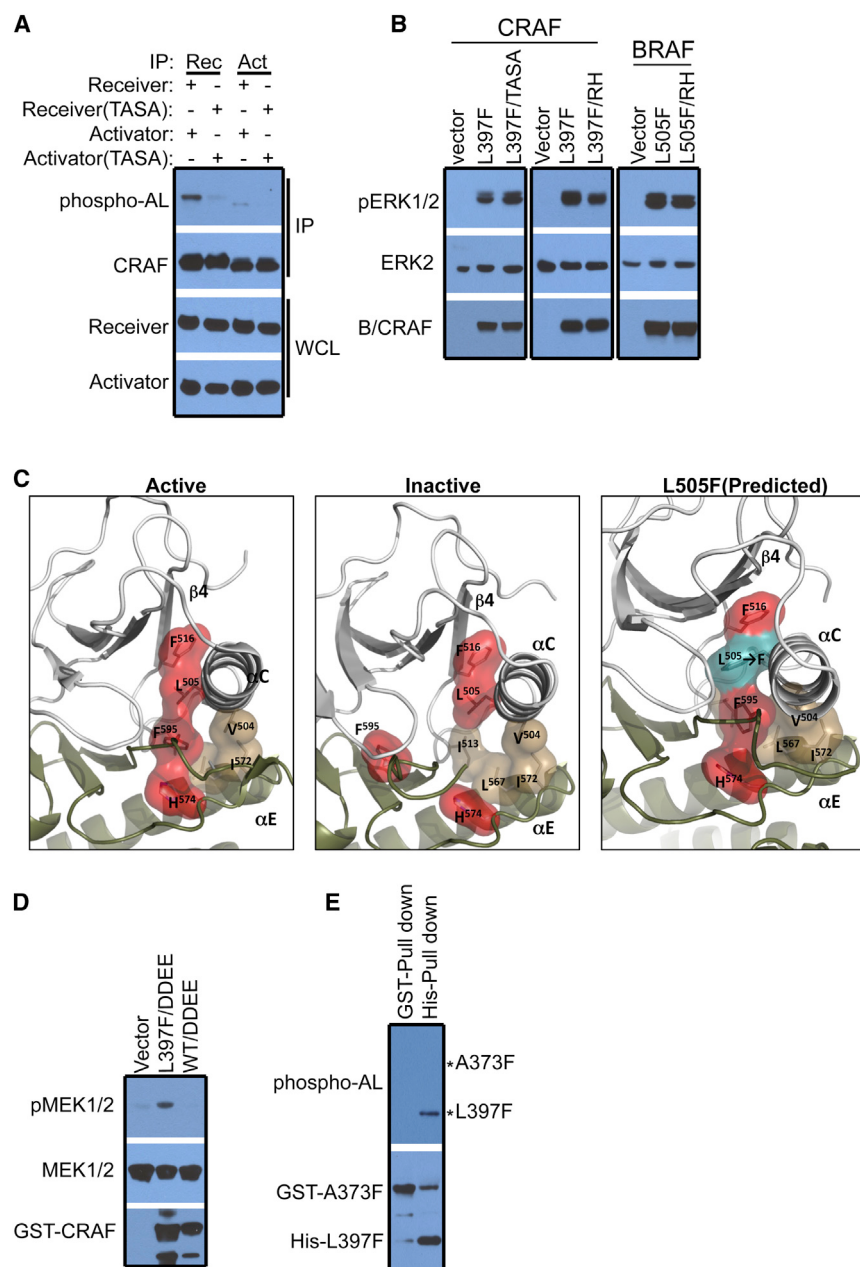


Figure 4. Constitutive Assembly of the R-Spine Results in an Active Kinase that Does Not Require Dimerization or AL Phosphorylation

(A) Phosphorylation of the AL is induced on the receiver but not the activator. An HA-tagged CRAF activator (A373F/DDEE, Δ 1–322) was coexpressed with a FLAG-tagged CRAF receiver (A373F/DDEE, Δ 1–322). 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 phosphospecific 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 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 (Protein Data Bank [PDB] ID code 4E26). In the middle panel, the position of the residues of the R-spine in the inactive, dimeric structure of BRAF is shown (PDB ID code 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 bacterially 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* toward purified MEK.

(E) Coexpression of His-tagged CRAF L397F/DDEE construct with GST-CRAF/A373F mutant in bacteria. Bacteria were lysed, and proteins purified with glutathione or Ni^{2+} beads and immunoblotted with BRAF pT599 antibody.

rather than, as currently believed, before activation. Pretreatment of cells with two different MEK inhibitors blocked a significant fraction of S338 phosphorylation induced by EGF (Figures 5A, 5B, and S2) or by BRAF inhibitor (Figure 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. Coexpression 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-

ures 5C and 5D). These data showing that CRAF S338 phosphorylation can occur as a consequence of CRAF activation suggest that it functions as a positive feedback mechanism allowing the activation of CRAF to convert the CRAF receiver into a CRAF activator (depicted in Figure 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

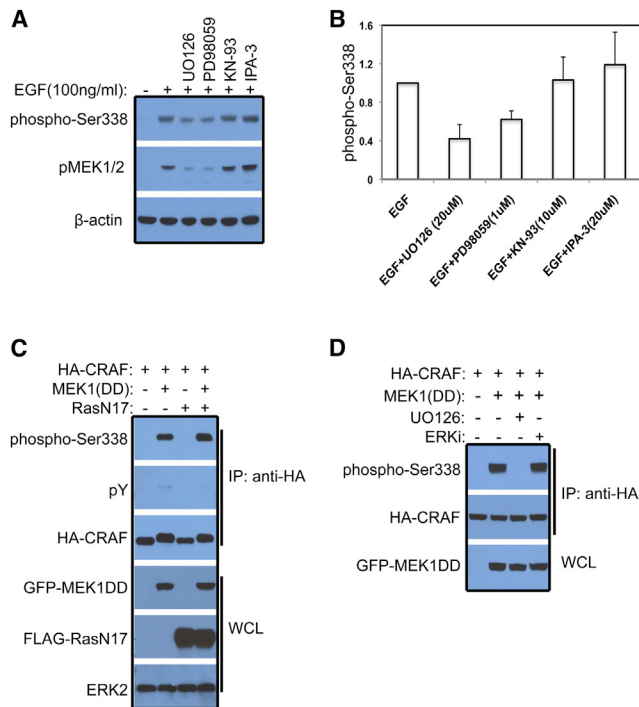


Figure 5. CRAF S338 Phosphorylation Is Stimulated by MEK Activation

(A) MEK inhibitors inhibit CRAF S338 phosphorylation induced by EGF. Untransfected 293 cells were pretreated with MEK inhibitor (UO126—20 μ M or PD98059—1 μ M), PAK inhibitor (IPA3—20 μ M), CAMKII inhibitor (KN-93—10 μ M), or vehicle for 2 hr before addition of EGF (100 ng/ml). Cells were lysed after 5 min, and CRAF S338 and MEK phosphorylation assessed using phosphospecific antibodies. See also Figure S2.

(B) Quantification of the effect of kinase inhibitors on CRAF S338 phosphorylation. Results from four separate experiments are presented as mean $\pm$ standard error of the mean (SEM). The amount of S338 phosphorylation from the EGF treatment alone was set as 1.

(C) Coexpression of constitutively active MEK with CRAF induces S338 phosphorylation. Wild-type HA-CRAF was coexpressed with constitutively active MEK1 (DD) with and without dominant-negative RAS (RasN17). Cells were lysed after 24 hr, and immunoprecipitates prepared with antibodies to HA. CRAF S338 phosphorylation was assessed using a phosphospecific antibody.

(D) MEK inhibition blocks CRAF S338 phosphorylation induced by coexpression with constitutively active MEK. Cells were prepared as described in (B), except that MEK (UO126) or ERK inhibitor (ERK inhibitor II) was added 24 hr after transfection. Cells were lysed 2 hr after addition of the inhibitor. Immunoprecipitates were analyzed as in (B).

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), PAKs, and PKCs (King et al., 1998; Kolch et al., 1993). For BRAF, these phosphorylations are unnecessary because 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, using a variety of different mutants (Table S1), that the function of the phosphorylated NtA motif is to facilitate transactivation, explaining how dimerization func-

tions to stimulate RAF kinase activity. Our data support a model wherein 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 constitutive phosphorylation of the NtA in BRAF accounts for its ability to transactivate and is a key functional difference between BRAF and CRAF. 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 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 previously generated mutant 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.

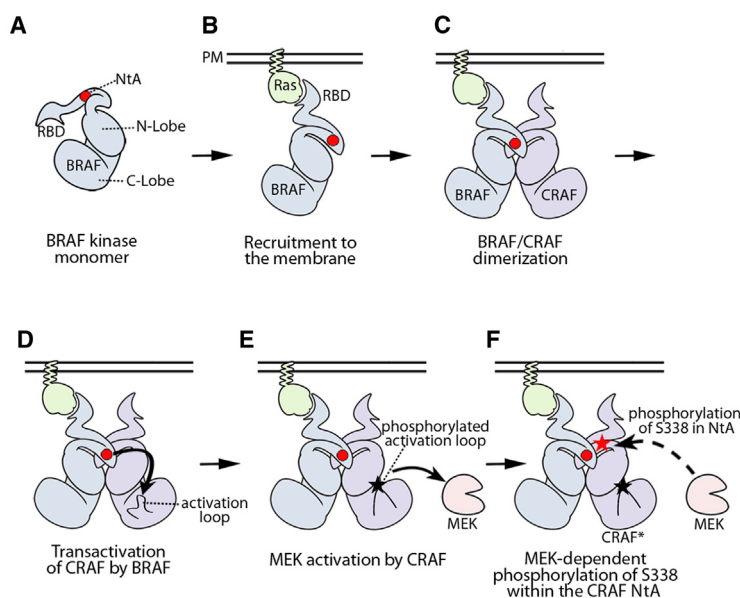
This difference between BRAF and CRAF suggests that the stoichiometry between BRAF and CRAF levels could be important. CRAF molecules are considered to be more abundant than BRAF molecules in fibroblasts (Mikula et al., 2001). We confirmed and quantitated this, showing that CRAF is about 3–4 times more abundant than BRAF in fibroblast cell lines (Figure 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 (Figure 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 minimized 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 (E.A.G., J.H., and A.S.S., unpublished data). Differences in activation thresholds could play an important role in determining the quality of MAPK 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 (Garrett et al., 2005; Hatzivassiliou et al., 2010; Poulikakos et al., 2010; Wan et al., 2004). We confirmed these findings (Garrett 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 (Figure S5). This suggests that the greater abundance of CRAF expression can explain the directionality of RAF activation.

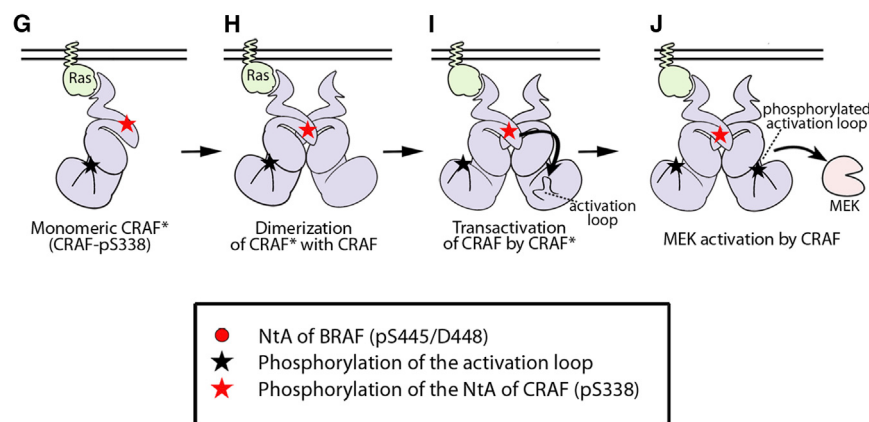
Mechanism of RAF Activation

Recently, we proposed a model wherein the assembly of two hydrophobic spines, the catalytic and the regulatory spines, can

BRAF activates CRAF (to generate CRAF*)



CRAF* activates CRAF



provide a unifying explanation for protein kinase activation (Kornev et al., 2008; Taylor and Kornev, 2011). 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 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 $\beta 4$ 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.

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 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 with CRAF (the receiver). CRAF* may also dimerize with, and activate, a molecule of BRAF. (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.

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 $\beta 4$ 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, 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 way that also contributes to inhibition of 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 (Figure 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 α C helix are all situated close to the dimer interface (Baljuls et al., 2011; Wan et al., 2004) (Figure S6).

AL phosphorylation is usually thought to occur in *trans* by a second kinase. In the RAF dimer, AL phosphorylation appears to occur by *cis*-autophosphorylation. The conundrum presented by *cis*-autophosphorylation is that the kinase must be primed for *cis*-autophosphorylation. Although the exact mechanism of *cis*-autophosphorylation is unknown, the process of such priming for RAF must be driven by dimerization. GSK-3 β , p38 α , 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. Because 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 (Figure 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). However, the V600E mutation only works for BRAF, whereas the R-spine mutation activates both BRAF and CRAF, supporting the broad applicability of the spine hypothesis in dissecting kinase activation. AL phosphorylation also occurred when the mutated CRAF was expressed in bacteria, supporting the idea that AL phosphorylation occurs by *cis*-autophosphorylation. The fact 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

(Figure 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 regarding 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 coworkers showed that BRAF dimerization with KSR2 could stimulate KSR2 kinase activity toward 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 ways to think about RAF isoforms and how their differences could be used to modulate signaling through the MAPK signaling pathway.

EXPERIMENTAL PROCEDURES

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 were from Thermal Scientific; anti-BRAF and anti-ERK2 were from Santa Cruz Biotechnology; and HRP-labeled secondary antibodies were 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^{-/-} mouse embryonic fibroblasts (MEFs) were described previously (Nguyen et al., 2002). The CRAF^{-/-} MEFs were purchased (Hüser 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 hr, and lysed in RIPA buffer supplemented with protease and phosphatase inhibitors.

Stable cell lines were generated by retro- or lentiviruses generated using 293T cells transfected with appropriate viral packaging vectors. Viral supernatants were collected at 48 and 72 hr and used to infect target cells, following standard protocols (Hu et al., 2008). Transduced cells were selected by 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 assays, the immunoprecipitates were washed with kinase reaction buffer (25 mM HEPES, 10 mM MgCl₂, 0.5 mM Na₃VO₄, 0.5 mM DTT, pH 7.4), then incubated with kinase reaction buffer

plus 2 μ g inactive MEK1 and 100 μ M ATP per sample at room temperature for 30 min. Kinase reaction was stopped by adding 5 μ l 4 $\times$ Laemmli sample buffer. Immunoblotting was carried out as described before (Hu et al., 2011).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2013.07.046>.

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