Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59795R3
Full Title:	Characterize disease-related RAF mutants by using a set of practical and feasible methods
Keywords:	RAF Kinase, RAF mutation, Ras/RAF/MEK/ERK signaling, Dimerization, Dimer affinity/stability, Catalytic activity, Allosteric activity, In vitro kinase assay, RAF co-activation assay, Complementary Split Luciferase Assay, Cancer.
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Singapore

TITLE:

Characterizing Disease-Related Mutants of RAF Family Kinases by Using a Set of Practical and Feasible Methods

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KEYWORDS:

RAF family kinases, RAF mutation, Ras/RAF/MEK/ERK signaling, dimerization, dimer affinity/stability, catalytic activity, allosteric activity, in vitro kinase assay, RAF co-activation assay, complementary split luciferase assay, cancer

SUMMARY:

In this article, we presented a set of practical and feasible methods for characterizing disease-related mutants of RAF family kinases, which include in vitro kinase assay, RAF co-activation assay, and complementary split luciferase assay.

ABSTRACT:

The rapidly accelerated fibrosarcoma (RAF) family kinases play a central role in cell biology and their dysfunction leads to cancers and developmental disorders. A characterization of disease-related RAF mutants will help us select appropriate therapeutic strategies for treating these diseases. Recent studies have shown that RAF family kinases have both catalytic and allosteric activities, which are tightly regulated by dimerization. Here, we constructed a set of practical and feasible methods to determine the catalytic and allosteric activities and the relative dimer affinity/stability of RAF family kinases and their mutants. Firstly, we amended the classical in vitro kinase assay by reducing the detergent concentration in buffers, utilizing a gentle quick wash procedure, and employing a glutathione S-transferase (GST) fusion to prevent RAF dimers from dissociating during purification. This enables us to measure the catalytic activity of constitutively active RAF mutants appropriately. Secondly, we developed a novel RAF co-activation assay to evaluate the allosteric activity of kinase-dead RAF mutants by using N-terminal truncated RAF proteins, eliminating the requirement of active Ras in current protocols and thereby achieving a higher sensitivity. Lastly, we generated a unique complementary split luciferase assay to quantitatively measure the relative dimer affinity/stability of various RAF mutants, which is more reliable and sensitive compared to the traditional co-immunoprecipitation assay. In summary, these methods have the following advantages: (1) user-friendly; (2) able to carry out effectively without advanced equipment; (3) cost-effective; (4) highly sensitive and reproducible.

INTRODUCTION:

The RAF family kinases are a key component of RAS/RAF/MEK/ERK signaling cascade, which transmit a signal from RAS to activate mitogen-activated protein kinase (MEK)[1-4]. This family of kinases plays a crucial role in cell growth, survival and differentiation, and their alterations induce many diseases, notably cancer[5-8]. Recently, genomic sequencings have identified many disease-related RAF mutants that exhibit different properties in the signal transmission of RAS/RAF/MEK/ERK cascade[9-11]. A careful characterization of RAF mutants will help us understand the molecular mechanisms of how RAF mutants alter the signal output of RAS/RAF/MEK/ERK cascade, eventually select appropriate approaches for treating various RAF mutant-driven diseases.

The RAF family kinases include three members, CRAF, BRAF, and ARAF, which have similar molecular structures but different abilities to activate downstream signaling[1-4]. Among these paralogs, BRAF has the highest activity by virtue of its constitutively phosphorylated NtA (N-terminal acidic) motif[12-14], while ARAF has the lowest activity arising from its non-canonical APE motif[15]. This may explain the different mutation frequencies of RAF paralogs in diseases: BRAF>CRAF>ARAF. Moreover, within the same RAF paralog, mutations in different sites may trigger downstream signaling in distinct manners, which adds another layer of complexity to the regulation of RAF family kinases. Recent studies have demonstrated that all RAF kinases have both catalytic and allosteric activities[13,14,16-18]. Constitutively active RAF mutants turn on the downstream signaling directly by phosphorylating MEK, whereas kinase-dead RAF mutants can transactivate their wild-type counterparts through side-to-side dimerization and activate MEK-ERK signaling[16,19,20]. The dimer affinity/stability is a key parameter that not only determines the allosteric activity of kinase-dead RAF mutants but also affects the catalytic activity of constitutively active RAF mutants[15,21,22]. The kinase-dead RAF mutants with high dimer affinity/stability can transactivate the endogenous wild-type RAFs directly[15], while those with intermediate dimer affinity/stability requires a coordination of active Ras or an elevated level of wild-type RAF molecules to function[13,15,20,21,23]. Similarly, constitutively active RAF mutants phosphorylate MEK in a dimer-dependent manner, and those with low dimer affinity/stability lose their catalytic activity in vitro upon immunoprecipitation that breaks the weak RAF dimers[15,21,22]. The dimer affinity/stability also determines the sensitivity of RAF mutants to their inhibitors, and positively correlates to the resistance of RAF inhibitors[24]. Therefore, to characterize disease-related RAF mutants, it is necessary to measure their catalytic and allosteric activities, and dimer affinity/stability.

In recent years, our laboratory and others have developed various methods to characterize RAF family kinases and their mutants. According to our laboratory and others' experience, we think that the following three assays have advantages in defining disease-related RAF mutants: (1) the in vitro kinase assay that can be carried out with ease to detect the catalytic activity of constitutively active RAF mutants[15]; (2) the RAF co-activation assay that is a reliable and convenient method to measure the allosteric activity of kinase-dead RAF mutants[13,15,21-23,25]; (3) the complimentary split luciferase assay that has much higher sensitivity in measuring the relative dimer affinity/stability of RAF mutants in contrast to the traditional co-

immunoprecipitation assay, and is able to carry out without advanced equipment in contrast to the quantitative analytic methods such as SPR (Surface Plasmon Resonance) analysis[15,22]. Combining these three assays, we can understand easily how a disease-related RAF mutant alters the downstream signaling and thereby utilize an appropriate therapeutic strategy to treat the disease caused by this RAF mutation.

PROTOCOL:

1. In vitro kinase assay for measuring the catalytic activity of RAF mutants

1.1. Construct vectors encoding RAF mutants (**Figure 1A**) with FLAG(DYKDDDDK) tag at C-terminus by using Gibson Assembly or traditional molecular cloning methods.

1.1.1. Introduce the FLAG tag and mutations into the RAF coding sequences by PCRs, and then insert whole sequences into pCDNA3.1(+) vector by using Gibson assembly or T4 DNA ligation and following the manufacture's protocols. Use the following conditions for PCR reactions: (1) 95 °C, 2 min; (2) 95 °C, 30 s; (3) 59 °C, 30 s; (4) 68 °C, 3 min; (5) 20 cycles of (2); (6) 4 °C hold.

NOTE: The PCR primers for cloning: 5- AAATTAATACGACTCACTATAGGGAGACCC-3 and 5-CAGCGGGTTTAAACGGGCCCTCTA-3.

1.1.2. Insert the GST coding sequence upstream of RAF mutant coding sequences to generate vectors encoding GST-fused RAF mutants by using same methods as described in step 1.1.1.

1.1.3. Validate all vectors by DNA sequencings before transfection.

1.2. Plate 293T cells in 6-well plates at a density of $5x10^5$ cells/well one day before transfection. When the cell density reaches 80~90% confluence on the second day, transfect with vectors encoding FLAG-tagged RAF kinases or their mutants from step 1.1 into cells by following the manufacture's protocol of transfection reagents (**Table of Materials**).

1.3. Replace the culture medium 24 h after transfection.

1.4. Aspirate the culture medium 48 h after transfection and add 400 µL/well of lysis buffer (25 mM Tris·HCl, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.25% NP-40, pH 7.2) supplemented with protease and phosphatase inhibitors to lyse cells on ice.

NOTE: The concentration of NP-40 in lysis buffer is critical for detecting the catalytic activity of RAF mutants with moderate dimer affinity/stability in vitro. A high concentration of detergent or a strong detergent in lysis buffer may break RAF dimers and thereby kill the catalytic activity of RAF kinases or their mutants.

1.5. Transfer the cell lysates to a 1.5 mL tube, and spin down by 12,000 x *g* for 10 min at 4 °C to deplete cell debris.

1.6. Transfer 300 µL per sample of clean whole cell lysates to 1.5 mL tubes, add 20 µL per sample of anti-FLAG affinity beads, and rotate in a cold room (4 °C) for 1 h. Also take 40 µL per sample of clean whole cell lysate aside for detecting the expression and activity (phospho-ERK1/2) of RAF mutants by immunoblots as described below.

1.7. Wash the anti-FLAG beads once with lysis buffer, then once with kinase reaction buffer (20 mM HEPES, 10 mM $MgCl_2$, 0.5 mM Na_3VO_4, 0.5 mM DTT, pH 7.2), and add 20 µL of kinase reaction mixture (2 µg of MEK1(K97A) and 100 µM ATP in 20 µL of kinase reaction buffer) per sample.

NOTE: The bead washing should be completed gently and quickly, the residual buffer should be aspirated completely before adding kinase reaction mixture, and all operations at this step should be carried out at 4 °C in a cold room.

1.8. Incubate the kinase reactions at room temperature (25 °C) for 10 min, and flip the tubes containing kinase reactions with fingers every other minute during incubation.

1.9. Add 5 µL of 5x SDS sample buffer (375 mM Tris·HCl, 9% sodium dodecyl sulfate (SDS), 50% Glycerol, 0.03% Bromophenol Blue) per sample to stop kinase reactions, and then heat the samples at 90 °C for 5 min.

1.10. Run the samples in 9~12% polyacrylamide gel electrophoresis (PAGE) with 0.1% SDS, transfer the proteins to nitrocellulose membrane, and detect the levels of phospho-MEK and RAF mutants in samples by immunoblots.

NOTE: The phospho-MEK can also be quantified by using γ^{32}P-ATP incorporation. Briefly, 10 µM γ^{32}P-ATP is added to the kinase reaction buffer, and the amount of phosphorylated MEK is then quantified after PAGE separation by using standard autoradiography, phosphorimaging, or liquid scintillation counting techniques as appropriate.

2. RAF co-activation assay for evaluating the allosteric activity of kinase-dead RAF mutants

2.1. Construct vectors encoding the RAF receiver (CRAF kinase domain with unphosphorylatable NtA motif, AAFF) or the kinase-dead RAF activators (RAF kinase domain with phosphorylation-mimicked NtA motif, SSDD, DDEE or DGEE) (**Figure 1A**) as described in step 1.1.

2.2. Transfect 293T cells with two vectors encoding both the RAF receiver and the kinase-dead RAF activator or a single vector encoding one of proteins as described in steps 1.2 and 1.3.

2.3. Replace the culture medium at 24 h after transfection, and harvest 293T transfectants at 48 h to prepare the whole cell lysates as described in steps 1.4 and 1.5.

2.4. Mix the clean whole cell lysate with 5x SDS sample buffer quickly at room temperature (25 °C) and then boil at 90 °C for 5 min.

2.5. Run the boiled whole cell lysate samples in 9~12% PAGE with 0.1% SDS, transfer the proteins to nitrocellulose membrane, and detect the levels of phospho-ERK1/2 and control proteins by immunoblots.

3. Complimentary split luciferase assay for measuring the relative dimer affinity/stability of RAF mutants.

3.1. Construct vectors encoding FLAG-tagged RAF mutants fused to the N-terminus of Nluc (N-terminus of firefly luciferase, aa2-416) or the C-terminus of Cluc (C-terminus of firefly luciferase, aa398-550) as described in step 1.1.

3.2. Transfect 293T cells with a pair of vectors encoding different Nluc-RAF mutants and Cluc-RAF mutants as described in step 1.2.

3.3. At 24 h after transfection, replate 293T cell transfectants into Krystal black image plates at the cell density of $2x10^5$ per well with color-free medium (i.e., DMEM without phenol red).

3.4. 24 h later, add D-luciferin (0.2 mg/mL) to 293T cell transfectants, incubate for 30 min, and measure the luciferase signals by using a multi detection system (**Table of Materials**).

3.5. After measuring the luciferase signals, aspirate the medium and lyse 293T transfectants with lysis buffer to prepare the whole cell lysates as described in steps 1.4 and 1.5.

3.6. Run the whole cell lysate samples in 9~12% PAGE with 0.1% SDS and detect the expression levels of Nluc-RAF mutants and Cluc-RAF mutants by anti-FLAG immunoblot as described in step 2.5. The relative expression levels of both Nluc-RAF mutant and Cluc-RAF mutant in 293T transfectants are quantified by using image J from their immunoblots.

3.7. Normalize the luciferase signals of 293T cell transfectants according to the expression levels of Nluc-RAF mutants and Cluc-RAF mutants. Briefly, this is achieved by dividing the raw luciferase signal by the relative expression levels of Nluc-RAF mutants and Cluc-RAF mutants from step 3.6.

REPRESENTATIVE RESULTS:

The RAF family kinases have both catalytic and allosteric activities, which enable their disease-related mutants to turn on the downstream signaling through different mechanisms[13,14,16-18]. The constitutively active RAF mutants directly phosphorylate their substrates, while the kinase-dead RAF mutants fulfill their function through transactivating wild-type counterparts. As shown in **Figure 1B**, both constitutively active RAF mutants (such as Regulatory spine (R-spine) mutants (BRAF(L505M), CRAF(DDEE/L397M), and ARAF(DGEE/L358M))[13,23,25,26], BRAF(V600E), and BRAF(ΔNVTAP)) and kinase-dead RAF mutants (such as Catalytic spine (C-spine)-fused

BRAF(ΔNVTAP/V471F)[15]) activates ERK when expressed in 293T cells. Therefore, the ability of RAF mutants to activate ERK signaling in cells cannot serve as a standard to distinguish a constitutively active mutant from a kinase-dead mutant, although some kinase-dead mutants with moderate dimer affinity/stability turns on downstream signaling only with the cooperation of active Ras. Three methods that we presented here can help us effectively characterize all disease-related RAF mutants. The biological properties of all RAF mutants revealed by using these assays in our previous studies have been summarized in **Table 1**.

The first method that we can use to distinguish the constitutively active RAF mutants from the kinase-dead RAF mutants is the in vitro kinase assay. In this assay, the RAF mutants were purified by immunoprecipitation and the catalytic reactions were carried out in vitro with kinase-dead MEK1(K97A) and ATP as substrates. The catalytic activity of RAF mutants was measured as the AL(Activation Loop)-phosphorylation of MEK1(K97A) in the kinase reaction mixtures by immunoblot. As shown in **Figure 1C**, this assay can effectively probe the catalytic activity of R-spine mutants of BRAF, CRAF and ARAF[13,15,23], BRAF(V600E)[9], and BRAF(ΔNVTAP)[15], but not that of kinase-dead BRAF(V471F/ΔNVTAP)[15] that has a fused C-spine. However, the catalytic activity of constitutively active RAF mutants with weak dimer affinity/stability such as ARAF R-spine mutant (ARAF(DGEE/L358M)) (**Figure 1C**, lane 4), CRAF and ARAF mutants with altered dimer interface (CRAF(DDEE/L397M/R401H), ARAF(DGEE/L358M/APE/R362H))[15,22] (**Figure 1D,E**) might not be probed by using this assay, since their dimers were broken during the purification, especially when the purification was carried out with buffers containing strong or high-concentration detergents. To avoid the loss of catalytic activity of RAF mutants with weak dimer affinity/stability, we usually fused these mutants with GST (glutathione S-transferase), a dimeric protein with strong affinity/stability before carrying out in vitro kinase assay, which can rescue the catalytic activity of these RAF mutants[15,22] (**Figure 1E**). In general, most RAF mutants could be classified as constitutively active or kinase-dead mutants by using this assay.

The second method that we described here is the RAF co-activation assay that can be used to evaluate the allosteric activity of kinase-dead RAF mutants[13,15,21-23,25]. Although a few kinase-dead RAF mutants with very high dimer affinity/stability such as BRAF(V471F/ΔNVTAP)[15], can directly activate endogenous RAF molecules when expressed in cells (**Figure 1B**, lane 7), most kinase-dead RAF mutants require the cooperation of active Ras to transactivate wild-type RAFs. However, active Ras is a direct activator of ERK signaling, whose introduction will increase the basal level of active ERK. To avoid the interference of active Ras, we used N-terminus-truncated RAF mutants in this assay (**Figure 2A**). As shown in **Figure 2B**, kinase-dead mutants of BRAF, CRAF, and RAF with acidic NtA motif and C-spine fusion (BRAF(V471F, aa431-766), CRAF(DDEE/V363F, aa323-648), and ARAF(DGEE/V324F, aa284-606)) functioned as allosteric activators to trigger the catalytic activity of CRAF with non-phosphorylatable NtA motif (CRAF receiver, CRAF(AAFF, aa323-648)) when co-expressed in 293T cells. In contrast, the kinase-dead BRAF mutant (V471F/ΔNVTAP, aa431-766) that have very high dimer affinity/stability (see below) turned on ERK signaling by triggering endogenous RAFs, even without the co-expression of CRAF receiver. Overall, this assay can be used to evaluate the transactivation ability of all kinase-dead RAF mutants.

The dimerization of RAF family kinases not only regulates their ability to activate downstream MEK-ERK signaling but also determines their sensitivity to RAF inhibitors[15,16,20,22,24,27-32]. In contrast to other protein-protein interactions among this pathway[33-35], the dimerization of RAF family kinases and their mutants is relatively weak and difficult to be evaluated by using traditional biochemistry assays such as co-immunoprecipitation. To resolve this problem, we recently developed a complimentary split luciferase assay for measuring the relative dimer affinity/stability of RAF family kinases and their mutants[15,22,36]. As shown in **Figure 3A**, the RAF kinase domains (aa431-766 for BRAF, aa323-648 for CRAF, and aa284-606 for ARAF) were fused respectively with N-terminus(aa2-416) or C-terminus(aa398-550) of firefly luciferase (Nluc and Cluc), which will be brought together to assemble intact luciferase upon RAF dimerization. The activity of luciferase in this assay directly correlates with the quantity of RAF dimers. This assay is very sensitive and can detect even a very weak dimerization of RAF mutants. As we reported before[15], the oncogenic BRAF(V600E) functions as a dimer, and only a compound mutation with the Arg-to-His mutation(R509H) in dimer interface and the non-canonic APE alteration(P622A in APE motif) can completely abolish its dimerization and thereby block its catalytic activity (Figure 3B). By using in vitro kinase assay, we further found that BRAF(V600E) variant with the altered APE motif, BRAF(V600E/AAE), but not that with the Arg-to-His mutation in dimer interface, BRAF(V600E/R509H), lost its catalytic activity upon purification (**Figure 3C**), suggesting that the former variant has much less dimer affinity/stability than the latter. To directly measure the propensity of these BRAF(V600E) variants to form dimers, we carried out a complimentary split luciferase assay, and confirmed that these variants had quite different dimer affinity/stability with BRAF(V600E) higher than BRAF(V600E/R509H) than BRAF(V600E/AAE) than BRAF(V600E/R509H/AAE) (**Figure 3D**). The luciferase signal produced by monomeric BRAF(V600E/R509H/AAE) was comparable with that of non-transfected 293T control (**Figure 3D**, left panel lane 5), indicating that this assay has a very low background. To further determine the effectiveness of complimentary split luciferase assay, we next measured by using this assay the relative dimer affinity/stability of a group of oncogenic BRAF mutants with in-frame deletions of β3- αC loop that was identified by us and other groups[15,37,38]. As we have known, although all of these mutants activated ERK signaling when expressed in 293T cells (**Figure 3E**), they exhibited quite differential catalytic activity in vitro (**Figure 3F**). The BRAF(ΔMLN(aa484-486 del)) and BRAF(ΔNVTAP(aa486-490 del)) were very active upon purification by immunoprecipitaion, whereas the BRAF(ΔNVTAPT(aa486-491 del)) and BRAF(ΔQA(aa496-497 del)) lost their catalytic activity under same condition though it could be rescued by GST fusion. In addition, the kinase-dead version of BRAF(ΔNVTAP), BRAF(V471F/ΔNVTAP) exhibited a strong potency to activate endogenous RAFs when expressed in cells (**Figure 1B,** lane 7). These data suggest that this group of BRAF mutants have quite different dimer affinity/stability with BRAF(ΔNVTAP) higher than BRAF(ΔMLN) than BRAF(ΔNVTAPT) and BRAF(ΔQA). Indeed, the result from the complimentary split luciferase assay completely supported our inference (**Figure 3G**). Taken together, our data indicate that the complimentary split luciferase assay is a reliable method to measure the relative dimer affinity/stability of RAF mutants.

FIGURE AND TABLE LEGENDS:

Figure 1. Probe the catalytic activity of RAF mutants by using in vitro kinase assay. (**A**) A

schematic diagram for RAF mutations used in this study. (**B**) Both constitutively active and kinase-dead RAF mutants can activate ERK signaling when expressed in cells. 293T cells were transfected with vectors that encode different RAF mutants, and the activity of ERK was detected by anti-phospho-ERK1/2 immunoblot. (**C**) Constitutively active RAF mutants with low dimer affinity/stability as well as kinase-dead RAF mutants cannot phosphorylate MEK in vitro upon purification by immunoprecipitation. RAF mutants in A were purified by using anti-FLAG beads, and their catalytic activity was probed by using in vitro kinase assay as described in the protocol. (**D-E**) The in vitro catalytic activity of constitutively active RAF mutants with low dimer affinity/stability can be rescued by GST fusion. (**D**) Constitutively active RAF mutants and their GST-fused counterparts were expressed in 293T cells and their activity was measured by anti-phospho-ERK1/2 immunoblot. (**E**) RAF mutants in **C** were purified by immunoprecipitation, and their in vitro catalytic activity was probed by using in vitro kinase assay as described in the protocol. RAF R-spine mutants: BRAF(L505M), CRAF(DDEE/L397M), and ARAF(DGEE/L358M). "ΔNVTAP" represents for the deletion of aa486-490 in BRAF. The APE mutation of ARAF means A475P that generates a canonical APE motif in ARAF. "KD" represents for "kinase domain" in the full text. BRAF(KD) means the aa431-766 fragment of BRAF, while CRAF(KD) and ARAF(KD) represent respectively for the aa323-648 fragment of CRAF and the aa284-606 fragment of ARAF. The results in this figure have been reported previously[15,22].

Figure 2. Evaluate the ability of RAF mutants to transactivate wild-type RAFs by using the RAF co-activation assay. (**A**) A diagram illustrating the RAF co-activation assay. (B) Kinase-dead RAF mutants with acidic NtA motif were co-expressed with catalysis-competent CRAF receiver in 293T cells, and the activity of ERK1/2 in 293T transfectants was measured by anti-phosho-ERK1/2 immunoblot. Most kinase-dead RAF mutants except BRAF(V471F/ΔNVTAP) that has a very high dimer affinity/stability required the co-expression of exogenous RAF receiver to turn on ERK signaling. The results in this figure have been reported previously[15,22].

Figure 3. **Measure the relative dimer affinity/stability of RAF mutants by using the complimentary split luciferase assay**. (**A**) A diagram illustrating the complimentary split luciferase assay. (B-D) The dimerization of BRAF(V600E) variants is required for their catalytic activity in vivo and in vitro. (**B**) The monomeric BRAF(V600E) variant, BRAF(V600E/R509H/AAE) has no catalytic activity in vivo, which can be recovered by GST fusion. BRAF(V600E) variants and their GST-fused counterparts were expressed in 293T cells, and their activity was measured by anti-phospho-ERK1/2. (**C**) The BRAF(V600E) variant with low dimer affinity/stability, BRAF(V600E/AAE) lost its catalytic activity in vitro upon purification, which can be rescued by GST fusion. BRAF(V600E) variants from **B** were purified by immunoprecipitation and their catalytic activity was probed by in vitro kinase assay as described in the protocol. (**D**) The relative dimer affinity/stability of BRAF(V600E) variants was measured by using the complimentary split luciferase assay as described in the protocol. (**E-G**) BRAF mutants with in-frame deletion of β3- αC loop function as dimers to activate ERK signaling. (**E**) BRAF mutants with in-frame deletion of β3- αC loop activate ERK signaling when expressed in cells. BRAF mutants were expressed in 293T cells and their activity was measured as described in **B**. (**F**) BRAF mutants with low dimer affinity/stability from E lost their catalytic activity in vitro, which can be rescued by GST fusion. The in vitro catalytic activity of BRAF mutants and their GST-

fused counterparts from **E** was measured as in **C**. (**G**) BRAF mutants with in-frame deletion of β3- αC loop have quite different dimer affinity/stability. The relative dimer affinity/stability of BRAF mutants with in-frame deletion of β3- αC loop was measured as in **D**. Error bars in D&G represent s.d. to show variance between independent experiments (n = 4). The AAE mutation of BRAF means P622A in APE motif that generates a non-canonical APE motif. "ΔMLN", "ΔNVTAPT", and "ΔQA" represent respectively for the deletions of aa484-486, aa486-491, aa496-497 in the β3- αC loop of BRAF. Some results in this figure have been reported previously[15].

Table 1. **The biological property of various RAF mutants.** The catalytic activity, allosteric activity and relative dimer affinity/stability of all RAF mutants used in this study have been summarized in this table. "Y" means "Yes", while "N" stands for "No". "N*" indicates that those mutants have catalytic activity if fused with GST. "++++", "+++", "++", "+", and "-" represent respectively for "very strong", "strong", "intermediate", "weak", and "none".

DISCUSSION:

In this article, we presented three methods for characterizing disease-related RAF mutants, which include in vitro kinase assay, RAF co-activation assay, and complimentary split luciferase assay. Since RAF kinases have both catalytic activity and allosteric activity, various RAF mutants can activate the downstream signaling through two distinct mechanisms[13,14,16-18]. The constitutively active RAF mutants directly phosphorylate the downstream effector MEK, whereas the kinase-dead RAF mutants trigger the downstream signaling through transactivating their wild-type counterparts. However, both constitutively active and kinase-dead RAF mutants require the dimerization to fulfill their function[15-17,19,20], and the dimer propensity of RAF mutants determines not only the catalytic or allosteric activity of RAF mutants but also their sensitivity to RAF inhibitors[15,24]. The in vitro kinase assay, RAF co-activation assay, and complimentary split luciferase assay provide us a set of simple, effective, and reliable methods for distinguishing the constitutively active mutants from the kinase-dead mutants as well as evaluating their ability to activate downstream signaling.

The in vitro kinase assay is a classical method to detect the catalytic activity of protein kinases. To adopt this method for measuring the catalytic activity of RAF mutants, we have made some significant revisions in reagents and procedures[15,21,22]. Since RAF family kinases function as dimers to phosphorylate their substrate, MEK, and their dimer affinity/stability is relatively low, we have reduced the concentration of detergent NP-40 from 1% to 0.25% in buffers for purifying RAF proteins in order to prevent RAF dimers from dissociation. By the same token, we have also used a gentle quick wash procedure for RAF protein purification. These changes are critical for detecting the catalytic activity of constitutively active RAF mutants with very weak dimer affinity/stability, which might be identified as "kinase-dead" RAF mutants by the classical in vitro kinase assay. In addition, we have demonstrated that the GST fusion is a good alternative way to prevent RAF dimers from dissociation during purification in this assay[15,21,22]. As to the kinase-dead RAF mutants, even if they are fused with GST, they do not exhibit any catalytic activity in this assay.

The RAF co-activation assay has been developed by us to evaluate the ability of kinase-dead RAF mutants to transactivate their wild-type counterparts[13,15,21-23,25]. In this novel assay, we use the N-terminus truncated RAF proteins and thereby do not need the co-expression of active RAS mutants or activating RAS, which makes this assay very sensitive. In addition, the allosteric activator (kinase-dead RAF mutant) and the receiver (catalysis-competent RAF mutant) in this assay can be easily isolated and purified for investigating molecular events in the process of dimerization-driven transactivation[13].

As we mentioned above, the dimer affinity/stability is a key factor that regulates the function of RAF family kinases and their mutants, and also determines their sensitivity to RAF inhibitors[13-18,24]. However, the dimer affinity/stability of RAF family kinases and their most disease-related mutants is very low. To measure this parameter, the traditional biochemistry assays require complicate experimental procedures and advanced equipment (such as SPR assay), or hardly produce reliable and reproducible data (such as immunoprecipitation assay). In contrast, the complimentary split luciferase assay is a simple, sensitive, consistent, and cost-effective method for measuring the relative dimer affinity/stability of RAF family kinases and their mutants[15,21,22,36]. This assay can probe the subtle difference of dimer affinity/stability among various RAF mutants. As we reported before[15], BRAF(V600E/AAE) has a very weak dimer affinity/stability and its dimers will be completely broken upon purification even if using a very gentle procedure. Using this assay, we can distinguish the weak dimer affinity/stability of BRAF(V600E/AAE) from that of monomeric BRAF(V600E/AAE/R509H) (**Figure 3**).

In summary, this set of practical and feasible methods can fulfill the requirements of defining all disease-related RAF mutants, and thereby help us select appropriate strategies for treating various RAF mutant-driven diseases.

ACKNOWLEDGMENTS:

The authors would like to acknowledge the Hairy Cell Leukemia Fellowship for support of Yuan Jimin. This work was supported by Asia Fund Cancer Research (AFCR2017/2019-JH), Duke-NUS Khoo Bridge Funding Award (Duke-NUS-KBrFA/2018/0014), NCCRF bridging grant (NCCRF-YR2018-JUL-BG4), NCCRF pilot grant (NCCRF-YR2017-JUL-PG3), and SHF Academic Medicine Research Grant (AM/TP011/2018).

DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

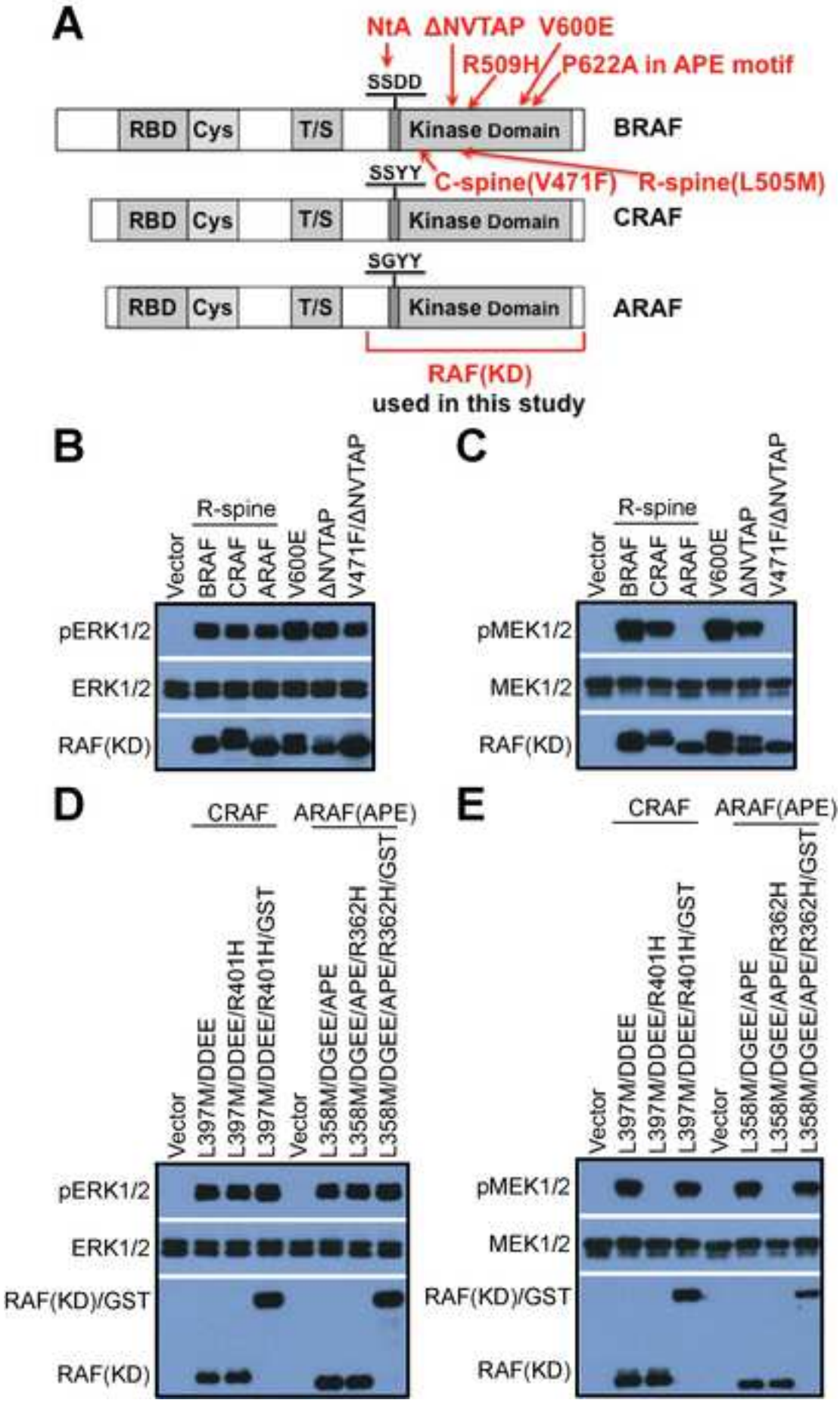
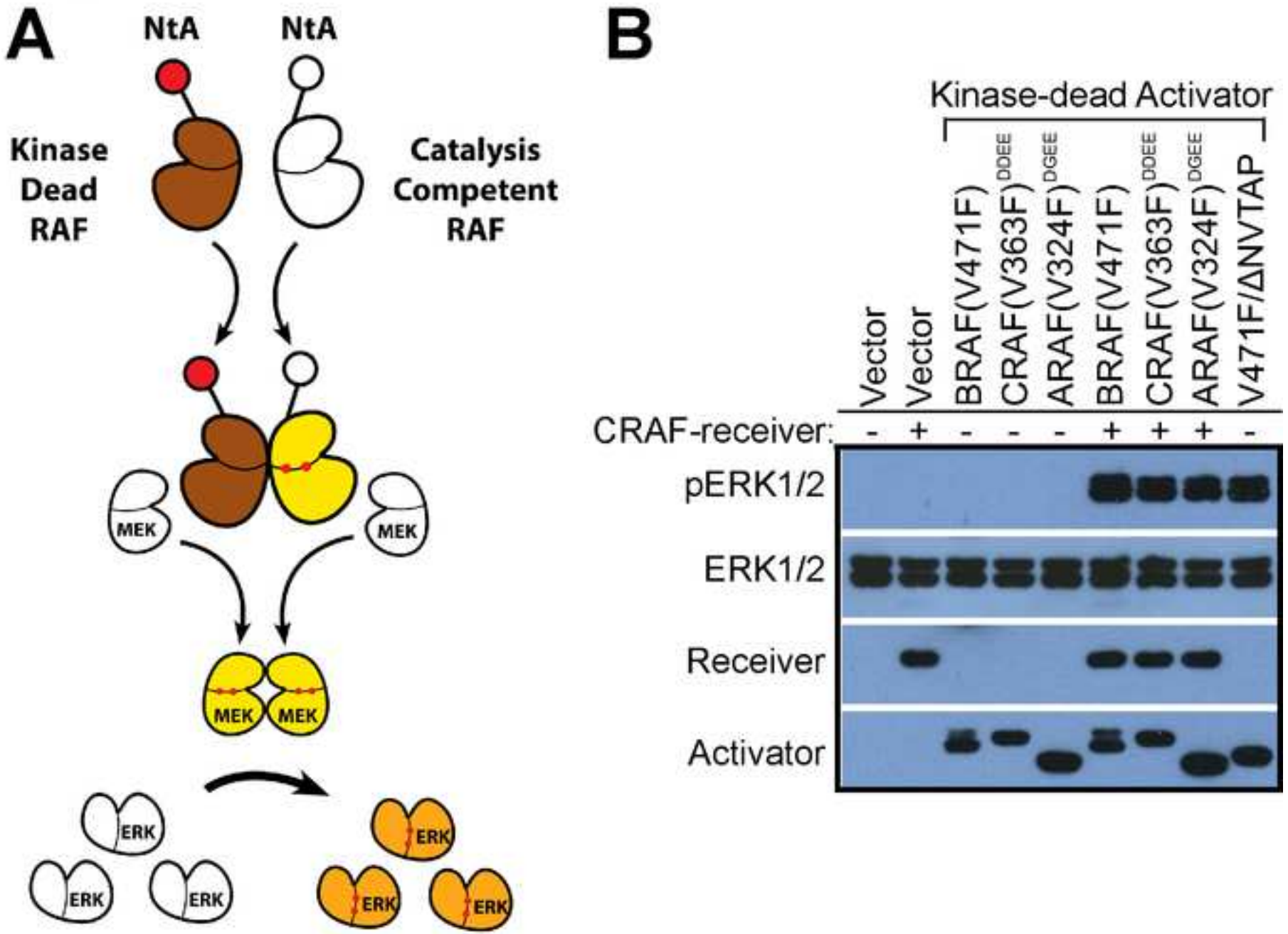

A
NtA ΔNVTAP V600E
R509H P622A in APE motif
SSDD
RBD Cys T/S Kinase Domain
BRAF
SSYY
C-spine(V471F) R-spine(L505M)
CRAF
SGYY
ARAF
RAF(KD)
used in this study
B
R-spine
Vector BRAF CRAF ARAF V600E ΔNVTAP V471F/ΔNVTAP
pERK1/2
ERK1/2
RAF(KD)
C
pMEK1/2
MEK1/2
D
CRAF ARAF(APE)
Vector L397M/DDEE L397M/DDEE/R401H L397M/DDEE/R401H/GST Vector L358M/DGEE/APE L358M/DGEE/APE/R362H L358M/DGEE/APE/R362H/GST
RAF(KD)/GST
E

Figure 2



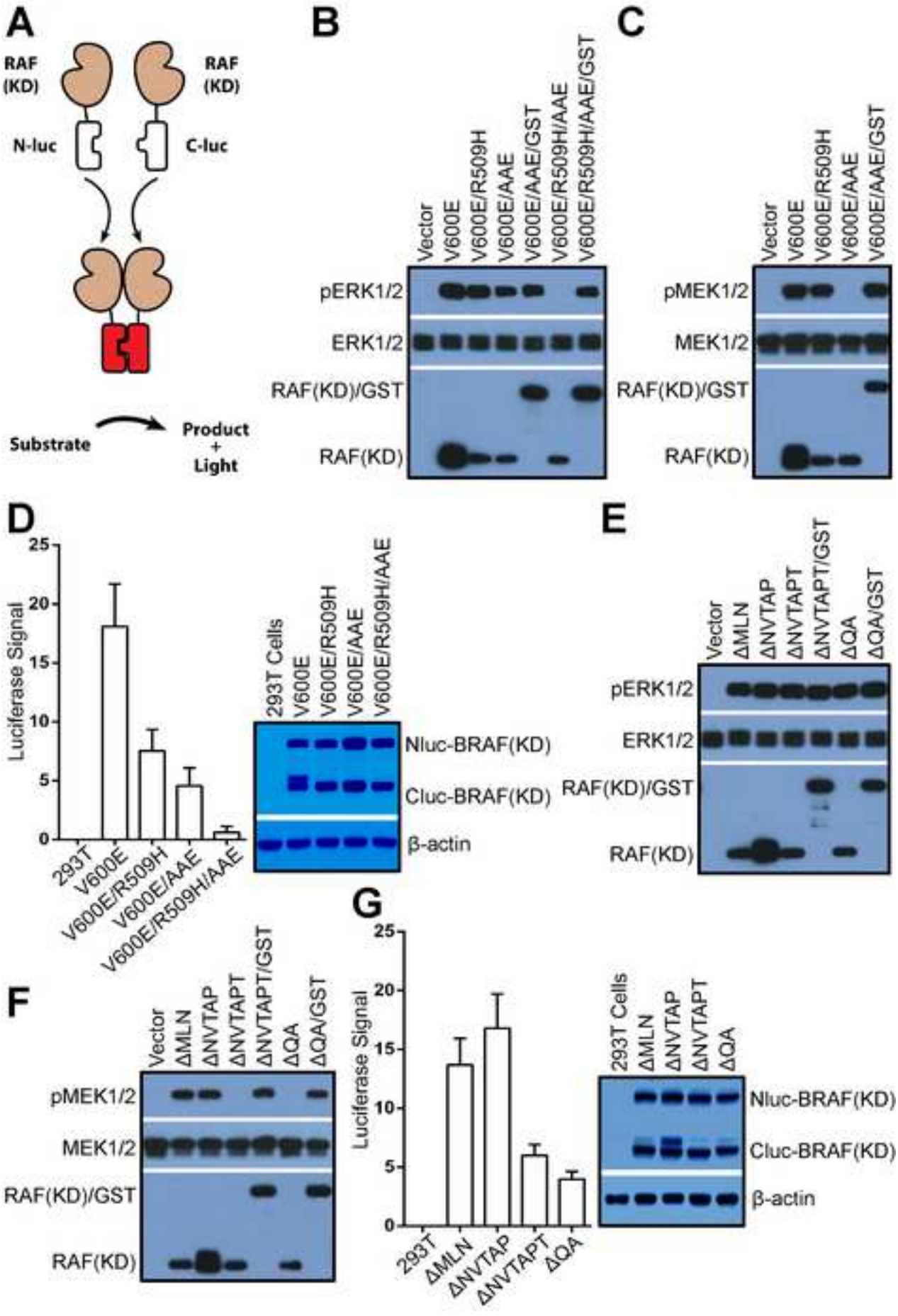


Figure 3
A
RAF (KD)
RAF (KD)
N-luc
C-luc
Substrate
Product + Light
B
Vector
V600E
V600E/R509H
V600E/AAE
V600E/AAE/GST
V600E/R509H/AAE
V600E/R509H/AAE/GST
pERK1/2
ERK1/2
RAF(KD)/GST
RAF(KD)
C
Vector
V600E
V600E/R509H
V600E/AAE
V600E/AAE/GST
pMEK1/2
MEK1/2
RAF(KD)/GST
RAF(KD)
D
Luciferase Signal
25
20
15
10
5
0
293T
V600E
V600E/R509H
V600E/AAE
V600E/R509H/AAE
293T Cells
V600E
V600E/R509H
V600E/AAE
V600E/R509H/AAE
Nluc-BRAF(KD)
Cluc-BRAF(KD)
β-actin
E
Vector
ΔMLN
ΔNVTAP
ΔNVTAPT
ΔNVTAPT/GST
ΔQA
ΔQA/GST
pERK1/2
ERK1/2
RAF(KD)/GST
RAF(KD)
F
Vector
ΔMLN
ΔNVTAP
ΔNVTAPT
ΔNVTAPT/GST
ΔQA
ΔQA/GST
pMEK1/2
MEK1/2
RAF(KD)/GST
RAF(KD)
G
Luciferase Signal
25
20
15
10
5
0
293T
ΔMLN
ΔNVTAP
ΔNVTAPT
ΔQA
293T Cells
ΔMLN
ΔNVTAP
ΔNVTAPT
ΔQA
Nluc-BRAF(KD)
Cluc-BRAF(KD)
β-actin

Table

RAF mutation	Constitutive activity	Kinase dead	Activated ERK signaling in cells	Activity *in vitro* (IVKA)
BRAF L505M	Y	N	Y	Y
BRAF V600E	Y	N	Y	Y
BRAF V600E R509H	Y	N	Y	Y
BRAF V600E AAE(P622A)	Y	N	Y	N*
BRAF V600E R509H AAE(P622A)	N	N*	N	N
BRAF ΔMLN	Y	N	Y	Y
BRAF ΔNVTAP	Y	N	Y	Y
BRAF ΔNVTAPT	Y	N	Y	N*
BRAF ΔQA	Y	N	Y	N*
BRAF V471F ΔNVTAP	N	Y	Y	N
CRAF DDEE L397M	Y	N	Y	Y
CRAF DDEE L397M R401H	Y	N	Y	N*
ARAF DGEE L358M	Y	N	Y	N*
ARAF DGEE L358M R362H	N	N*	N	N
ARAF DGEE L358M APE(A475P)	Y	N	Y	Y
ARAF DGEE L358M APE(A475P) R362H	Y	N	Y	N*

Dimer Affinity/Stability
+++
+++
++
+
-
+++
++++
+
+
++++
++
+
+
-
++
+

Table of Materials

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
anti-phosphoERK1/2	Cell Signaling Technologies	4370	
anti-phosphoMEK1/2	Cell Signaling Technologies	9154	
anti-ERK1/2	AB clonal	A0229	
anti-MEK1/2	Cell Signaling Technologies	9124	
anti-FLAG(mouse)	Sigma-Aldrich	F3165	
anti-HA	Novus Biologicals	MAB6875	
anti-FLAG(Rabbit)	Cell Signaling Technologies	14793	
anti-β-actin	Sigma-Aldrich	A2228	
anti-FLAG beads(M2)	Sigma-Aldrich	A4596	
HRP-conjugated anti-mouse IgG	Jackson Laboratories	115-035-003	
HRP-conjugated anti-Rabbit IgG	Jackson Laboratories	111-035-144	
pcDNA3.1(+)	In vitrogen	V79020	
Gibson Assembly Cloning Kit	New England Biolabs	E5510	
T4 DNA ligase	New England Biolabs	M0202	
lipofectamine 2000	Invitrogen	11668019	
Fugene 6	Roche	11 814 443 001	
DMEM w/o phenol red	Invitrogen	21063-029	
D-luciferin	GoldBio	LUCK-100	
6xhis-tagged MEK1 (K97A)	prepared in our previous studies	N.A.	Reference 15.
GloMax-Multi Detection System.	Promega	E7041	

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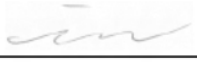
Signature:	
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