



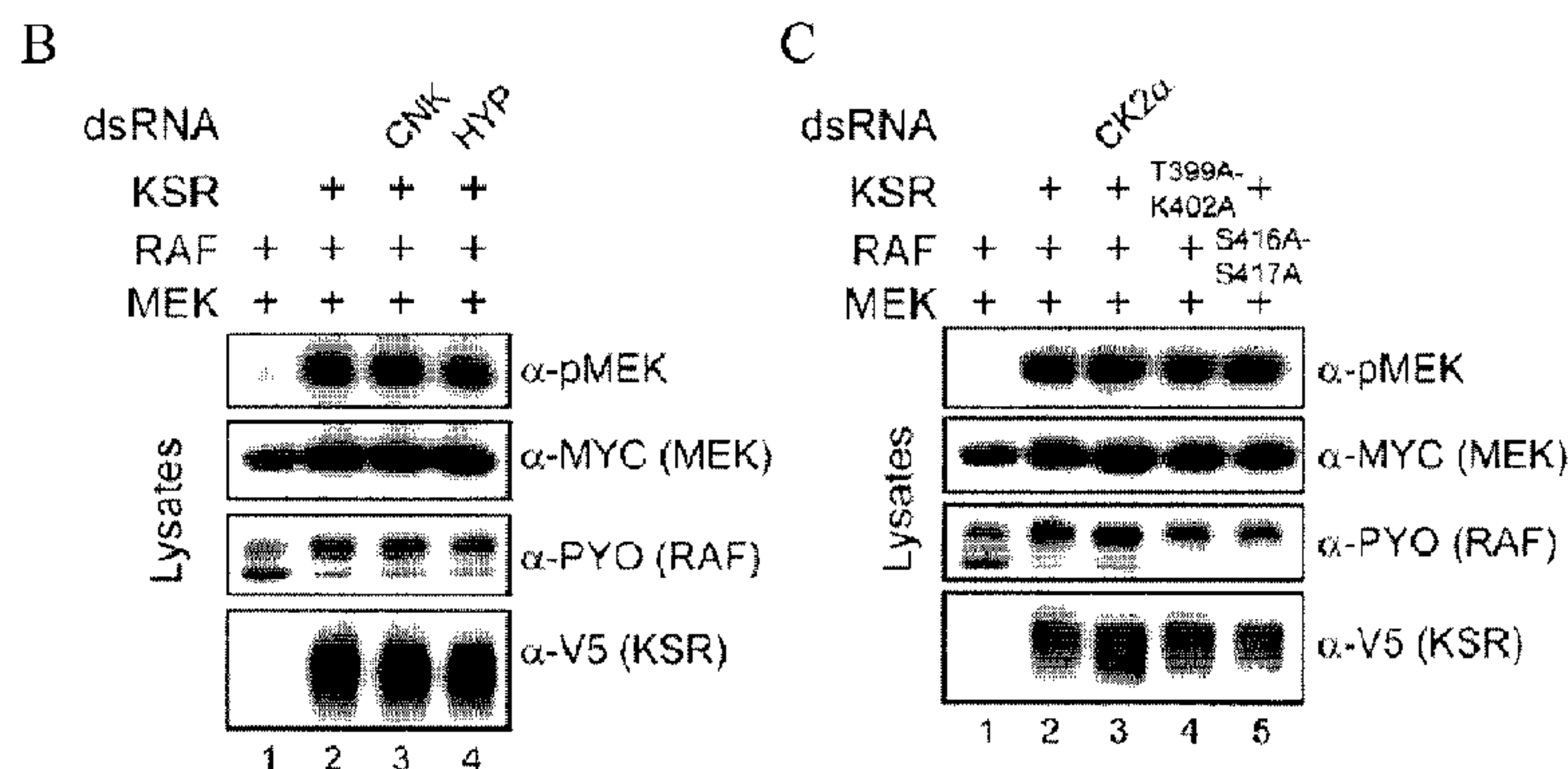
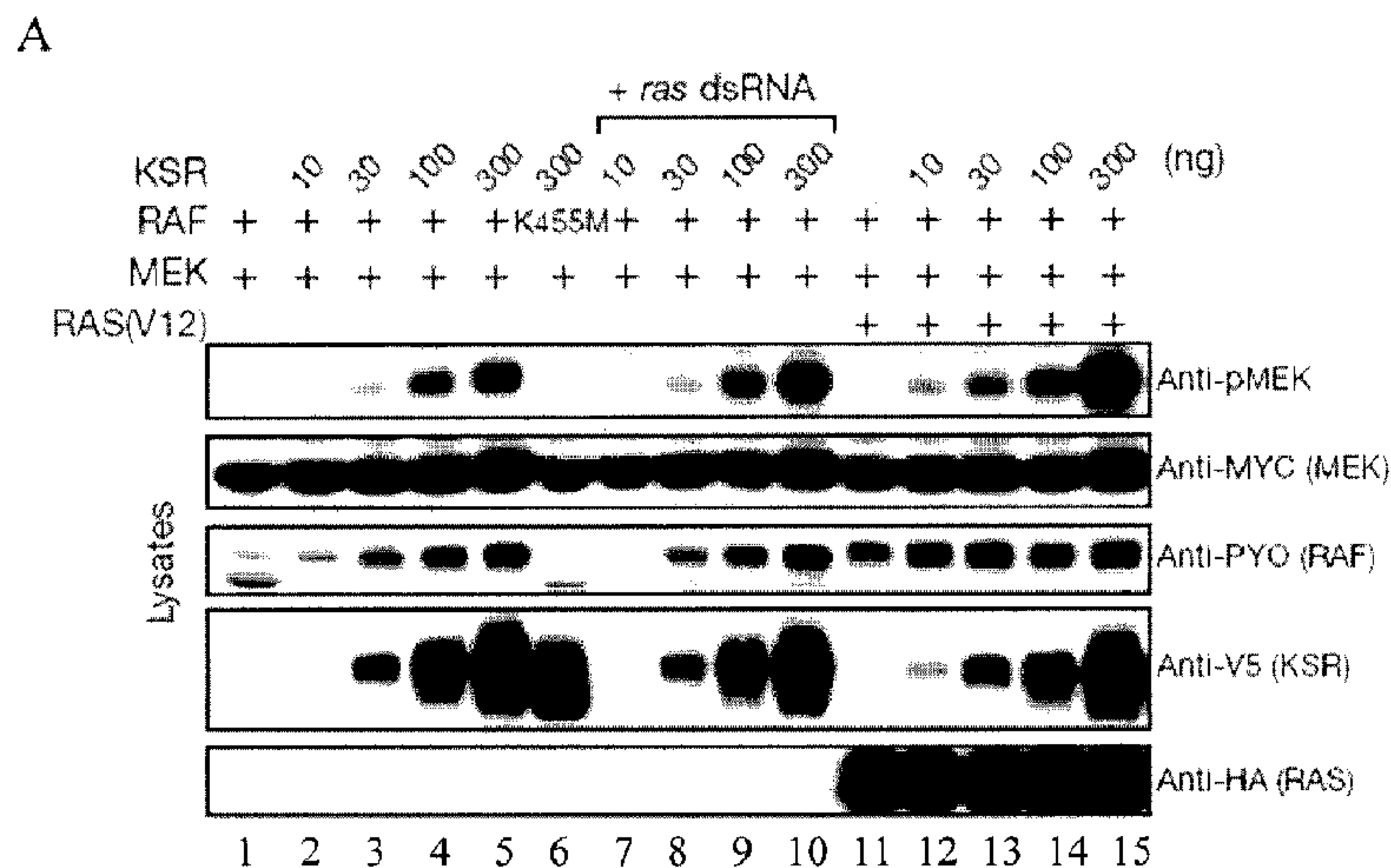
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(54) **Titre : BIOCAPTEURS FONDES SUR LE BRET ET METHODE DE SURVEILLANCE DE LA DIMERISATION DE RAF**
(54) **Title: BRET-BASED BIOSENSORS AND METHODS FOR MONITORING RAF DIMERIZATION**



(57) **Abrégé/Abstract:**

Disclosed herein are mutated RAF and KSR nucleic acids and polypeptides. Also disclosed are methods of using the mutated RAF and KSR to inhibit the dimerization of RAF/RAF and RAF/KSR. Also disclosed are methods of using the mutated RAF and KSR to



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(57) **Abrégé(suite)/Abstract(continued):**

screen for inhibitors of dimerization. Also disclosed are Bioluminescence Resonance Energy Transfer (BRET)-based biosensors comprising cells expressing RAF polypeptides fused to a BRET donor or acceptor, as well as methods using these biosensors for monitoring RAF dimerization.

ABSTRACT

Disclosed herein are mutated RAF and KSR nucleic acids and polypeptides. Also disclosed are methods of using the mutated RAF and KSR to inhibit the dimerization of RAF/RAF and RAF/KSR. Also disclosed are methods of using the mutated RAF and KSR to screen for inhibitors of dimerization. Also disclosed are Bioluminescence Resonance Energy Transfer (BRET)-based biosensors comprising cells expressing RAF polypeptides fused to a BRET donor or acceptor, as well as methods using these biosensors for monitoring RAF dimerization.

BRET-BASED BIOSENSORS AND METHODS FOR MONITORING RAF DIMERIZATION

TECHNICAL FIELD

The present generally concerns mutated RAF and KSR isoforms, and more particularly to their inhibition
5 of RAF/RAF and RAF/KSR dimer formation.

BACKGROUND

The ERK (extracellular signal-regulated kinase) pathway is an evolutionarily conserved signal
transduction module that controls cellular growth, differentiation and survival (Wellbrock, C.,
Karasarides, M. & Marais, R. The RAF proteins take centre stage, *Nat Rev Mol Cell Biol.*, 5, 875-85
10 (2004)). Activation of receptor tyrosine kinases (RTKs) by the binding of growth factors initiates GTP
loading of RAS, which triggers the initial steps in the activation of the ERK pathway by modulating RAF
family kinase function. Once activated, RAF participates in a sequential cascade of phosphorylation
events that activate MEK, and in turn ERK. Unbridled signaling through the ERK pathway caused by
activating mutations in RTKs, RAS or RAF, have been linked to a multitude of human cancers (Roberts,
15 P. J. & Der, C. J., Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the
treatment of cancer, *Oncogene*, 26, 3291-310 (2007)). Of note, one member of the RAF family, B-RAF,
is the most frequently mutated oncogene within the kinase superfamily (Greenman, C. et al., Patterns of
somatic mutation in human cancer genomes, *Nature*, 446, 153-8 (2007)).

Not surprisingly, there has been a colossal effort to understand the underlying regulation of this family of
20 kinases. Despite intense scrutiny, the mechanisms governing RAF activation remain only partially
understood. In particular, the process by which its kinase domain becomes catalytically activated towards
its substrate MEK remains elusive.

A greater understanding of the mechanisms that govern RAF activation would be useful as a means to
identify novel therapeutic intervention strategies for disease such as cancer.

25 BRIEF SUMMARY

The following addresses the shortcomings of the above.

In one aspect, there is provided a composition comprising: an aqueous solution of RAF/RAF homodimer.
The composition includes equimolar amounts of RAF monomers. Each RAF monomer includes a RAF
kinase domain having a dimerization interface. The RAF/RAF homodimer is a side-to-side dimer having
30 a 2 fold axis of symmetry.

In one aspect, there is provided a composition comprising: an aqueous solution of RAF/KSR heterodimer.
The composition includes equimolar amounts of KSR and RAF monomers. The KSR and the RAF

and the RAF monomer each include a kinase domain having a dimerization interface. The heterodimer has a 2-fold axis of symmetry.

In one aspect, there is provided a substantially pure nucleic acid encoding a mutated RAF polypeptide. The nucleic acid is DNA which contains the RAF gene. The DNA is genomic DNA or cDNA. The mutated RAF polypeptide is mutated A-RAF, mutated B-RAF or mutated C-RAF. The mutated RAF polypeptide includes at least one mutated residue located in a dimerization interface. The mutated residue is selected from the group consisting of: H449, G450, R481H, L487, F488, M489, Y538, A541 and K542. The mutation is selected from the group consisting of: H449E, G450W, R481H, L487R, F488A, F488L, M489W, Y538F, A541E and K542E. The mutated RAF polypeptide comprises a sequence of SEQ ID NO. 7, SEQ ID NO. 9, or SEQ ID NO. 15. The nucleic acid comprises a sequence of SEQ ID NO. 8, SEQ ID NO. 10 or SEQ ID NO. 16. The nucleic acid is DNA which is operably linked to regulatory sequences for expression of a mutated RAF polypeptide and wherein the regulatory sequences comprise a promoter. The promoter is a constitutive promoter, is inducible by one or more external agents, or is cell-type specific.

In another aspect, there is provided a method of producing a mutated RAF polypeptide, the method comprising:

- a) providing a cell transfected with a nucleic acid sequence encoding a mutated RAF polypeptide positioned for expression in the cell;
- b) culturing the transfected cells under conditions for expressing the nucleic acid; and
- c) producing the mutated RAF polypeptide.

In another aspect, there is provided a substantially pure mammalian mutated RAF polypeptide, or fragment thereof, the polypeptide being encoded by the nucleic acid, as described above. The polypeptide comprises an amino acid sequence substantially identical to an amino acid sequence of SEQ ID NO. 7 or SEQ ID NO. 9. The polypeptide includes at least one mutant selected from the group consisting of: H449E, G450W, R481H, L487R, F488A, F488L, M489W, Y538F, A541E and K542E.

In one aspect, there is provided a substantially pure nucleic acid encoding a mutated KSR polypeptide. The nucleic acid is DNA which contains the KSR gene. The DNA is genomic DNA or cDNA. The mutated KSR polypeptide is mutated KSR-1 or KSR-2. The mutated KSR polypeptide includes at least one mutated residue located in a dimerization interface. The mutated residue is selected from the group consisting of: H699, G700, R732, L738, F739, M740, Y790, A793 and R794. The mutation is selected from the group consisting of: H699E, G700W, R732H, L738R, F739A, F739L, M740W, Y790F, A793E and R794E. The mutated KSR polypeptide comprises a sequence of SEQ ID NO. 11, SEQ ID NO. 13, or SEQ ID NO. 17. The nucleic acid comprises a

sequence of SEQ ID NO. 12, SEQ ID NO. 14 or SEQ ID NO. 18. The nucleic acid is DNA which is operably linked to regulatory sequences for expression of the polypeptide and wherein the regulatory sequences comprise a promoter. The promoter is a constitutive promoter, is inducible by one or more external agents, or is cell-type specific.

5 In another aspect, there is provided a method of producing a mutated KSR polypeptide, the method comprising:

a) providing a cell transfected with a nucleic acid sequence encoding a mutated KSR polypeptide positioned for expression in the cell;

b) culturing said transfected cells under conditions for expressing the nucleic acid; and

10 c) producing the mutated KSR polypeptide.

In another aspect, there is provided a substantially pure mammalian mutated KSR polypeptide, or fragment thereof, the polypeptide being encoded by the nucleic acid, as described above. The polypeptide comprises an amino acid sequence substantially identical to an amino acid sequence of SEQ ID NO. 11, SEQ ID NO. 13 or SEQ ID NO. 17. The polypeptide includes at least one
15 mutant selected from the group consisting of: H699E, G700W, R732H, L738R, F739A, F739L, M740W, Y790F, A793E and R794E.

The polypeptide or the nucleic acid, as described above is mammalian. The mammal is murine or human.

The polypeptide or the nucleic acid, as described above, is non-mammalian. The non-mammal is
20 *Drosophila melanogaster*.

In another aspect, there is provided a vector comprising the nucleic acid, as described above, the vector being capable of directing expression of the polypeptide encoded by the nucleic acid in a vector-containing cell.

In another aspect, there is provided a cell that contains the nucleic acid, as described above.

25 In another aspect, there is provided a transgenic cell that contains the nucleic acid, as described above, wherein the nucleic acid is expressed in the transgenic cell.

In another aspect, there is provided a transgenic non-human mammal generated from the cell, as described above, wherein the nucleic acid is expressed in the transgenic mammal. The transgenic non-human mammal is a mouse.

30 The cell, as described above, is a mammalian cell, a yeast cell, or a bacterial cell.

In one aspect, there is provided a method of detecting the presence of a mutation in a RAF kinase domain, the method comprising:

a) providing a WT RAF kinase domain and a suspected mutant RAF kinase domain, each domain having a cysteine residue located at its N-terminus;

5 b) incubating the WT RAF kinase domain and the suspected mutant RAF kinase domain with different cross-linking detectable labels;

c) incubating together equimolar amounts of the labeled WT RAF kinase domain and detecting a signal from the detectable label so as to provide a dimerization reference signal; and

10 d) incubating equimolar amounts of the labeled suspected mutant B-RAF kinase domain and detecting a signal from the detectable labels, an absent signal or a reduce signal compared to that of the dimerization reference signal being an indication that a mutant B-RAF kinase domain is present.

15 In one aspect, there is provided a method of monitoring the formation of RAF/RAF or RAF/KSR kinase domain dimers to detect mutations inhibiting dimerization or drug-like molecules interfering with dimerization, the method comprising:

a) fusing either (i) a RAF kinase domain or (ii) a KSR kinase domain at either of their N- or C-termini to a BRET donor or a BRET acceptor to produce donor labeled and acceptor labeled fusion proteins;

20 b) expressing the fusion proteins to identify combinations that provide specific BRET signals;

c) introducing dimer interface mutations into either of the labeled fusion proteins;

d) expressing the labeled mutated fusion proteins with WT RAF or KSR kinase domains;

25 e) measuring the BRET signals, a loss or significant reduction of the BRET signal using dimer interface mutations as opposed to mutations remote from the interface, being an indication that a specific BRET signal which depends on the RAF/RAF or RAF/KSR dimerization interface has been obtained.

30 The BRET donor is *renilla* luciferase variant II or rlucII. The BRET acceptor is GFP10. The acceptor label is Yellow Fluorescent Protein (YFP). The donor labeled fusion protein comprises a sequence selected from the group consisting of: SEQ ID NO. 24, SEQ ID NO. 34, SEQ ID NO. 42 and SEQ ID NO. 48. The acceptor labeled fusion protein comprises a sequence selected from the group consisting of: SEQ ID NO. 22, SEQ ID NO. 30, SEQ ID NO. 40 and SEQ ID NO. 54. The

donor labeled mutated fusion proteins comprise sequences SEQ ID NO. 36 and SEQ ID NO. 50. The acceptor labeled mutated fusion proteins comprises a sequence of SEQ ID NO. 32.

In another aspect, there is provided a method of identifying a potential inhibitor of RAF/RAF homodimerization, the method comprising.

- 5 a) fusing a RAF kinase domain at either of its N- or C-termini to a BRET donor or a BRET acceptor to produce donor labeled and acceptor labeled fusion proteins;
- b) expressing the fusion proteins to identify combinations that provide specific BRET signals;
- c) introducing dimer interface mutations into either of the labeled fusion proteins;
- 10 d) expressing the labeled mutated fusion proteins with WT RAF kinase domains;
- e) contacting the interface with the potential inhibitor; and
- f) measuring the BRET signals, a loss or significant reduction of the BRET signal for the wild-type RAF/RAF BRET pair being an indication that the inhibitor is specifically bound to the interface.
- 15 In another aspect, there is provided a method of identifying a potential inhibitor of RAF/RAF homodimerization, the method comprising:
 - a) detectably labeling at least one of the dimerization interface residues to generate a detectably labeled RAF monomer;
 - b) incubating the detectably labeled RAF monomer with the potential inhibitor and a non-20 labeled RAF monomer;
 - c) measuring a signal from the detectable label;
 - d) contacting the RAF dimerization interface with the inhibitor to determine the ability of the potential inhibitor to inhibit RAF/RAF homodimerization.

The interface residues include H449, G450, R481, L487, F488, M489, Y538, A541 or K542.

- 25 In one aspect, there is provided a method of identifying a potential inhibitor of RAF/RAF homodimerization, the method comprising.

- a) fusing a RAF kinase domain at either of its N- or C-termini to a BRET donor or a BRET acceptor to produce donor labeled and acceptor labeled fusion proteins;

b) expressing the fusion proteins to identify combinations that provide specific BRET signals;

c) introducing dimer interface mutations into either of the labeled fusion proteins;

d) expressing the labeled mutated fusion proteins with WT RAF kinase domains;

5 e) contacting the interface with the potential inhibitor; and

f) measuring the BRET signals, a loss or significant reduction of the BRET signal for the wild-type RAF/RAF BRET pair being an indication that the inhibitor is specifically bound to the interface.

10 In another aspect, there is provided a method of identifying compounds that bind to a RAF or a KSR dimerization interface, the method comprising:

a) contacting the interface with a probe to form a probe: interface complex, the probe being displaceable by a test compound;

b) measuring a signal from the probe so as to establish a reference level;

c) incubating the probe:interface complex with the test compound;

15 d) measuring the signal from the probe;

e) comparing the signal from step d) with the reference level, a modulation of the signal being an indication that the test compound binds to the BIR domain, wherein the probe is a compound labeled with a detectable label or an affinity label.

20 In another aspect, there is provided a method of identifying a potential inhibitor of RAF/RAF homodimerization, the method comprising:

a) using the atomic coordinates of at least one of the interface residues to generate a three dimensional structure of a RAF dimerization interface;

b) using the three-dimensional structure to design or select the potential inhibitor;

c) synthesizing the inhibitor; and

25 d) contacting the RAF dimierization interface with the inhibitor to determine the ability of the potential inhibitor to inhibit RAF/RAF homodimerization.

The interface residues are H449, G450, R481, L487, F488, M489, Y538, A541 or K542.

In one aspect, there is provided a method of identifying a potential inhibitor of RAF/KSR heterodimerization, the method comprising:

a) using the atomic coordinates of at least one of interface residues to generate a three dimensional structure of a KSR dimerization interface;

5 b) using the three-dimensional structure to design or select the potential inhibitor;

c) synthesizing the inhibitor; and

d) contacting the KSR dimerization interface with the inhibitor to determine the ability of the potential inhibitor to inhibit RAF/KSR heterodimerization.

The interface residues are H699, G700, R732, L738, F739, M740, Y790, A793 or R794.

10 In another aspect, there is provided a method of detecting in a subject the susceptibility to develop a condition or an increased likelihood of developing a condition characterized by impaired regulation of protein RAF or KSR dimerization, the method comprising:

a) obtaining from said subject a biological sample having DNA;

15 b) sequencing predetermined regions of said DNA encoding a RAF or KSR polypeptide; and

c) comparing the sequence obtained at (b) with a corresponding sequence from a non-susceptible control subject for identifying a RAF or KSR mutation known to be indicative of the susceptibility.

20 In another aspect, there is provided a human RAF or KSR polypeptide which comprises a mutation compared to wild type RAF or KSR, wherein said mutation produces a mutant version of human RAF or KSR polypeptide that includes at least one mutant H449, G450, R481, L487, F488, M489, Y538, A541 or K542 residue, and wherein the mutant version prevents the formation of a RAF/RAF homodimer.

25 In another aspect, there is provided a human RAF kinase domain which comprises a mutated dimerization interface having at least one mutant H449, G450, R481H, L487, F488, M489, Y538, A541 or K542 residue.

In another aspect, there is provided a human KSR kinase domain which comprises a mutated dimerization interface having at least one mutant H699, G700, R732, L738, F739, M740, Y790, A793 or R794 residue.

In another aspect, there is provided a substantially pure nucleic having the sequence of full length RAF and encoding the polypeptide sequences of SEQ ID NO. 7 and SEQ ID NO. 9.

In another aspect, there is provided a substantially pure nucleic acid having about 50% or greater nucleotide sequence identity to the sequences, as described above.

- 5 In another aspect, there is provided a substantially pure nucleic having the sequence of full length KSR and encoding the polypeptide sequences of SEQ ID NO. 11, SEQ ID NO. 13 and SEQ ID NO. 17.

L0 In another aspect, there is provided a substantially pure nucleic acid having about 50% or greater nucleotide sequence identity to the sequences of SEQ ID NO. 12, SEQ ID NO. 14 and SEQ ID NO. 18.

In another aspect, there is provided a cell *in vitro* expressing a recombinant nucleic acid comprising a nucleic acid sequence encoding a mutated RAF polypeptide, as described above. In one example, the cell is a mammalian cell, a yeast cell, or a bacterial cell.

- 15 In one aspect, there is provided a method of producing a drug which inhibits RAF/RAF homodimerization, the method comprising: identifying a drug or designing a drug which interacts with at least one of the H449, G450, R481, L487, F488, M489, Y538, A541 and K542 residues; and synthesizing the drug.

- 20 In another aspect, there is provided a method of producing a drug which inhibits RAF/KSR heterodimerization, the method comprising: identifying a drug or designing a drug which interacts with at least one of the H699, G700, R732, L738, F739, M740, Y790, A793 and R794 residues; and synthesizing the drug.

- 25 In another aspect, there is provided a composition comprising: an inhibitor adapted to inhibit the formation of a RAF/RAF homodimer or a RAF/KSR heterodimer, in which the inhibitor binds to at least one of the H449, G450, R481, L487, F488, M489, Y538, A541 and K542 residues in a RAF monomer or at least one of the H699, G700, R732, L738, F739, M740, Y790, A793 and R794 residues in a KSR monomer.

- 30 In another aspect, there is provided a method of treating or preventing a disease in a subject, the disease being characterized by RAF/RAF homodimerization or RAF/KSR heterodimerization, the method comprising: administering to the subject in need thereof, an expression vector encoding mutated RAF or KSR polypeptide, the mutated RAF or KSR polypeptide being positioned in the vector for expression in a cell of the subject in which RAF/RAF homodimerization or RAF/KSR heterodimerization is taking place, so as to treat or prevent the disease.

In another aspect, there is provided a purified antibody which specifically binds to a mammalian mutated RAF or KSR polypeptide. The mammal is a human. The mammal is a mouse. The mutated RAF polypeptide is B-RAF. The KSR polypeptide is KSR-1. The antibody is a polyclonal antibody. The antibody is a monoclonal antibody.

- 5 In another aspect, the present invention provides a biosensor comprising:
an isolated human embryonic kidney (HEK) cell expressing:
(a) a first fusion molecule comprising the amino acid sequence of SEQ ID NO: 34; and
(b) a second fusion molecule comprising the amino acid sequence of SEQ ID NO: 30.
- 10 In another aspect, the present invention provides a method for determining whether a test agent modulates the homodimerization of human BRAF, the method comprising:
a) providing the biosensor defined herein in the presence or absence of said test agent; and
b) measuring the fluorescence signal emitted by said second fusion molecule in said cell,
wherein a higher fluorescence signal measured in the presence of the test agent is indicative that
15 said test agent increases the homodimerization of human BRAF, and a lower fluorescence signal measured in the presence of the test agent is indicative that said test agent inhibits the homodimerization of human BRAF.

BRIEF DESCRIPTION OF THE FIGURES

20 In order that the herein described may be readily understood, certain embodiments are illustrated by way of example in the accompanying drawings.

Figure 1 – KSR possesses intrinsic RAF activating potential. A) Co-overexpression of KSR, RAF and its substrate MEK as indicated in S2 cells leads to activation of RAF in a KSR concentration-dependent manner in the presence or absence of RNAi-mediated knockdown of RAS or co-overexpression of a constitutively active RAS^{V12} variant. RAF activation was assessed by
25 immunoblotting for phospho-MEK. The catalytically-inactive RAF K455M (KM) mutant served as a negative control. B and C) The RAF activation potential of overexpressed KSR is not affected by RNAi-mediated knockdown of CNK, HYP or CK2 α or by mutation of the proposed CK2 α regulatory sites in KSR (T399A/K402A) and RAF (S416A/S417A). Assessment of the RNAi-mediated knock-downs for endogenous RAS, CNK, HYP and CK2 α is provided in Figure 13.

30 **Figure 2 – KSR_R732H mutation abolishes its inherent RAF activating potential.** Wild type KSR but not the KSR_R732H mutant is able to drive RAF activation in an S2 cell overexpression system. Experiments were performed as in Figure 1.

Figure 3 – A side-to-side dimer configuration of RAF underlies an allosteric mode of regulation. A) Projection of highly conserved residues across both KSR and RAF orthologues onto the crystal structure (PDB ID = 1UWH) (Wan, P. T. et al., Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF, Cell, 116, 855-67 (2004)) of the B-RAF kinase domain (top panel) highlights common side-to-side dimer contact surfaces visualized originally in crystal structures of B-RAF (bottom panel). B) Crystal structure of B-RAF highlighting the position of Arg481 (equivalent to Arg732 in KSR) at the center of the side-to-side dimer interface (PDB ID = 1UWH) (Wan, P. T. et al., Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF, Cell, 116, 855-67 (2004)). Residue numbering scheme corresponds to Drosophila RAF. One protomer is displayed as a surface representation in orange and the other is shown as a ribbons representation in violet. Inset displays a close-up view of hydrogen bonding interactions involving Arg481, an ordered solute molecule, and main-chain carbonyl groups in the linker joining helix α C to strand β 4.C) Analytical ultracentrifugation analysis reveals that mutation of

Arg481 (R481H) in B-RAF transitions the protein from a dimer (left panel) to a monomer (right panel) in solution. The red line denotes a fitted curve to the self-association model. The residuals for the fit are shown in the upper panels.

Figure 4 – Side-to-side dimer interface residues are conserved in all KSR and RAF proteins.

5 Sequence alignment of the kinase domains of KSR and RAF from divergent organisms highlighting conserved residues. For comparison, the sequence of the kinase domains of LCK and PKA are co-aligned demonstrating that the side-to-side dimer contact residues are unique to the KSR/RAF family. The sequence of the kinase domain N-lobe is boxed in red and the secondary structural elements are indicated above the sequence. Aligned sequences correspond to those from
10 *Drosophila* (d), human (h), mouse (m), zebrafish (z), and chicken (c). Only the B-RAF sequence is shown for species where multiple RAF isoforms exist.

Figure 5 – Perturbing the side-to-side dimer interface on RAF and KSR impairs RAF activation.

A) Left panel: Model of a side-to-side heterodimer between KSR and RAF kinase domains. RAF is displayed as a surface representation in purple while KSR is shown in ribbons
15 representations in green. Highlighted in red stick representation are the positions of residues selected for mutational analysis in KSR (G700W, R732H, F739A, M740W and Y790F). Position of analogous mutated sites in RAF (G450W, R481H, F488A, M489W and Y538F, respectively) are denoted by yellow surface; residue numbering scheme corresponds to *Drosophila* RAF. Right panel: The individual effect of KSR and RAF mutations on RAF activation was assessed by
20 monitoring the levels of phosphorylated MEK in S2 cells as performed in Figure 1. Control mutations outside the side-to-side dimer interface correspond to K460A, E601A and M640A in RAF, and D710A, E859A and V898A in KSR. B) Left panel: Schematic for induced side-to-side dimer formation using FRB/FKBP fusions to the kinase domains of KSR and RAF. Right panel: The RAF activation potential of the FRB/FKBP fused kinase domains of KSR and RAF were assessed
25 by monitoring the levels of phosphorylated MEK in the presence or absence of rapamycin in S2 cells as performed in Figure 1. C) Left panel: Schematic for induced side-to-side homodimer formation of RAF kinase domains. The catalytically-inactive (K455S) FRB-RAF fusion is indicated by an 'X' within the N-lobe. Right panel: Activation potential of FRB/FKBP RAF homodimers was assessed as in Figure 3B.

30 **Figure 6 – The kinase domain of RAF adopts a side-to-side dimeric configuration in the crystal structure.** A) The side-to-side dimer configuration of the kinase domain of B-RAF is shown viewed perpendicular to the 2-fold axis of symmetry (PDB ID = 1UWH) (Wellbrock, C., Karasarides, M. & Marais, R. The RAF proteins take centre stage. *Nat Rev Mol Cell Biol* 5, 875-85 (2004)). The N-lobes of the two kinase domains, which compose the majority of the dimer
35 interaction surfaces, are colored in darker tint. B) Superposition of the six reported B-RAF kinase domain structures reveal an identical mode of side-to-side dimerization (PDB IDs: 1UWH (Wan, P. T. et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of

B-RAF. Cell 116, 855-67 (2004)), 1UWJ (Wan, P. T. et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell 116, 855-67 (2004)), 2FB8 (King, A. J. et al. Demonstration of a genetic therapeutic index for tumors expressing oncogenic BRAF by the kinase inhibitor SB-590885. Cancer Res 66, 11100-5 (2006)), 3C4C (Tsai, J. et al. Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity. Proc Natl Acad Sci U S A 105, 3041-6 (2008)), 3C4D (Tsai, J. et al. Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity. Proc Natl Acad Sci U S A 105, 3041-6 (2008)) and 3D4Q (Hansen, J. D. et al. Potent and selective pyrazole-based inhibitors of B-Raf kinase. Bioorg Med Chem Lett 18, 4692-5 (2008)). C) Comparison of the B-RAF side-to-side mode of dimerization with the specific mode of dimerization of PKR (PDB ID = 2A19 (Dar, A. C., Dever, T. E. & Sicheri, F. Higher-order substrate recognition of eIF2 α by the RNA-dependent protein kinase PKR. Cell 122, 887-900 (2005)) and EGFR (PDB ID = 2GS2 (Zhang, X., Gureasko, J., Shen, K., Cole, P. A. & Kuriyan, J. An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. Cell 125, 1137-49 (2006)) kinase domains. Helix α C is a participant in all three modes of dimerization.

Figure 7 – An engineered KSR/RAF chimera can functionally mimic wildtype KSR. A) Schematics of wild type and chimeric KSR/RAF constructs involving either a full RAF kinase domain swap into KSR (*Chimera-A*) or just a RAF N-lobe swap into KSR (*Chimera-B*). B) The ability of wild type RAF, KSR, and KSR/RAF chimeric constructs to drive RAF activation in S2 cells was assessed by the levels of phosphorylated MEK.

Figure 8 – Perturbing the side-to-side dimer interface on RAF and KSR impairs RAF activation. A) Model of a side-to-side heterodimer between KSR and RAF kinase domains. RAF is displayed as a surface representation in purple while KSR is shown in ribbons representations in green. Highlighted in red stick representation are the positions of residues selected for mutational analysis in KSR (H699E, L738R, F739L, A793E, and R794E). Position of analogous mutated sites in RAF (H449E, L487R, F488L, A541E and K542E, respectively) are denoted by yellow surface; residue numbering scheme corresponds to *Drosophila* RAF. B and C) The RAF activation potential of the FRB/FKBP fused kinase domains of KSR and RAF were assessed by monitoring the levels of phosphorylated MEK in the presence or absence of rapamycin in S2 cells as illustrated in the schematic.

Figure 9 – KSR contains a putative 14-3-3 binding site C-terminal to its kinase domain. A) Sequence alignment of the C-terminus of KSR reveals a highly conserved 14-3-3 recognition site common to that found in RAF (*Drosophila* RAF residue numbering is indicated above the alignment). Aligned sequences correspond to those from *Drosophila* (d), human (h), mouse (m), zebrafish (z), and chicken (c). B) S2 cell overexpression assay for RAF activation showing the effects of RNAi-mediated knockdown of 14-3-3 isoforms or mutation of putative 14-3-3 binding

sites in KSR (R950A/S951A) and RAF (S701A). The effect of RNAi on endogenous 14-3-3 protein levels is shown in Figure 13.

Figure 10 – Binding of 14-3-3 to KSR and RAF may promote the formation of hetero- and/or homotypic dimers by the kinase domain. Structural model showing that the geometry of the KSR/RAF (or RAF/RAF) side-to-side dimer is compatible with the spatial requirements for binding to dimeric 14-3-3 proteins. Surface representation of 14-3-3 bound to phospho-peptides is based on PDB ID 1YWT (Wilker, E. W., Grant, R. A., Artim, S. C. & Yaffe, M. B. A structural basis for 14-3-3sigma functional specificity. J Biol Chem 280, 18891-8 (2005)).

Figure 11 – Side-to-side dimer formation underlies the aberrant signaling potential of oncogenic RAF mutants. A) RAF activation assay using overexpressed full-length RAF and MEK proteins in S2 cells. The dimer interface mutation (RAF_R481H) abrogates the pronounced activation potential of the activation segment mutation (analogous to oncogenic B-RAF mutation) RAF_T571E/T574D (denoted RAF-AL^{ED}). B) Glu558 locates to the side-to-side dimer interface in RAF and is mutated to Lys in human cancers (E558K mutation; residue numbering scheme corresponds to *Drosophila* RAF). The longer Lys residue could potentially engage in hydrogen bonding interactions with Ser561 on the opposite protomer. C) *Left panel:* Schematic for induced side-to-side homodimer formation of RAF kinase domains as in Figure 3C. *Right panel:* Catalytically inactive FRB-RAF harboring the E558K mutation was assessed for its activation potential towards FKBP-RAF *in trans* as in Figure 3C.

Figure 12 – Oncogenic B-RAF E558K mutation promotes kinase domain dimerization. Analytical ultracentrifugation analysis reveals that the oncogenic E558K mutation in B-RAF transitions the B-RAF_L487R dimer mutant from weak monomer-dimer equilibrium (left panel) to a dimer (right panel) in solution; residue numbering scheme corresponds to *Drosophila* RAF. The red line denotes a fitted curve to the self-association model. The residuals for the fit are shown in the upper panels.

Figure 13 – Depletion of specific endogenous targets by RNAi. To ensure that dsRNAs directed against RAS, CNK, HYP, CK2 α , 14-3-3 ϵ or 14-3-3 ζ worked as expected, we separately incubated S2 cells with specific dsRNAs (15 μ g/ml) against these intended targets and monitored their respective protein or mRNA levels using either specific antibodies or qPCR. GFP dsRNA was used as negative control. In panel A, the effect of RAS depletion was also monitored by assessing phospho-MAPK (pMAPK) levels induced by the activated Sevenless (SEV^{S11}) RTK expressed under the control of the *hsp70* promoter (Laberge, G., Douziech, M., & Therrien, M. Src42 binding activity regulates *Drosophila* RAF by a novel CNK-dependent derepression mechanism. EMBO J 24, 487-98 (2005)).

Figure 14A shows polypeptide and polynucleotide sequences (SEQ ID NO. 7 and 8) of homo sapiens v-raf murine sarcoma viral oncogene homolog B1 (BRAF) showing mutated residues as underlined and highlighted.

Figure 14B shows polypeptide and polynucleotide sequences (SEQ ID NO. 9 and 10) of mus musculus Braf transforming gene (Braf) showing mutated residues as underlined and highlighted.

Figure 14C shows polypeptide and polynucleotide sequences (SEQ ID NO. 11 and 12) of homo sapiens kinase suppressor of ras 1 (KSR 1) showing mutated residues as underlined and highlighted.

Figure 14D shows polypeptide and polynucleotide sequences (SEQ ID NO. 13 and 14) of mus musculus kinase suppressor of ras 1 (Ksr 1) showing mutated residues as underlined and highlighted

Figure 14E shows polypeptide and polynucleotide sequences (SEQ ID NO. 15 and 16) of Drosophila melanogaster pole hole (phi) transcript variant A showing mutated residues as underlined and highlighted.

Figure 14F shows polypeptide and polynucleotide sequences (SEQ ID NO. 17 and 18) of Drosophila melanogaster kinase suppressor of ras (ksr) showing mutated residues as underlined and highlighted.

Figure 15 - Development of a Bioluminescence Resonance Energy Transfer (BRET) assay to monitor RAF/RAF homodimerization. (A) Structure of the human BRAF kinase. RBD, CRD and Ser/Thr stand for Ras-Binding Domain, Cysteine-Rich Domain and Ser/Thr-rich domains respectively. The Kinase domain (KD) and its C-terminal extension (dashed box) were used in all BRET constructs described here. (B) Structure of the BRAF-KD donor (rlucII) and acceptor (GFP10) expression constructs used in the BRET assay. (C) Saturation curve of the BRAF-KD-wt and BRAF-KD-R481H alleles showing a significant reduction in the BRET_{max} and BRET₅₀ when a dimer interface mutation (R481H) is introduced in the BRAF-KD. (D) Parameters derived from fit of our data with a hyperbolic function. R² denotes the goodness of fit of our data to a hyperbolic function. BRET_{max} and BRET₅₀ were interpolated using the hyperbolic function.

Figure 16 shows CAAX-box and BRET donor and acceptor polypeptide sequences (human KRAS CAAX-box CDS: SEQ ID NO. 19; human KRAS CAAX-box: SEQ ID NO. 20; GFP10 CDS: SEQ ID NO. 21; GFP10: SEQ ID NO. 22; rlucII CDS: SEQ ID NO. 23; and rlucII: SEQ ID NO. 24).

Figure 17 shows human BRAF (hBRAF) polypeptide sequences (hBRAF-KD-wt CDS: SEQ ID NO. 25; hBRAF-KD-wt: SEQ ID NO. 26; hBRAF-KD-R481H CDS: SEQ ID NO. 27; hBRAF-KD-R481H:

SEQ ID NO. 28). The bolded residues indicate linker and restriction sites. Mutated residues are shaded in black.

Figure 18 shows human BRAF-KD clones between the NheI and XbaI sites in pCDNA3.1-zeo (GFP10-hBRAF-KD-wt-CAAX CDS: SEQ ID NO. 29; GFP10-hBRAF-KD-wt-CAAX: SEQ ID NO.30; GFP10-hBRAF-KD-R481H-CAAX CDS: SEQ ID NO. 31; GFP10-hBRAF-KD-R481H-CAAX: SEQ ID NO. 32; rlucII-hBRAF-KD-wt-CAAX CDS: SEQ ID NO. 33; rlucII-hBRAF-KD-wt CAAX: SEQ ID NO. 34; rlucII-hBRAF-KD-R481H-CAAX CDS: SEQ ID NO. 35; and rlucII-hBRAF-KD-R481H-CAAX: SEQ ID NO. 36). The bolded residues indicate linker and restriction sites. Mutated residues are shaded in black.

Figure 19 shows human CRAF (hCRAF) polypeptide sequences (hCRAF-KD-wt CDS: SEQ ID NO. 37; hCRAF-KD-wt: SEQ ID NO. 38).

Figure 20 shows hCRAF-KD fusions that are cloned between NheI and XbaI in pCDNA3.1-zeo (GFP10-hCRAF-KD-wt-CAAX CDA: SEQ ID NO. 39; GFP10-hCRAF-KD-wt-CAAX: SEQ ID NO. 40; rlucII-hCRAF-KD-wt-CAAX CDS: SEQ ID NO. 41; and rlucII-hCRAF-KD-wt-CAAX: SEQ ID NO. 42). The bolded residues indicate linker and restriction sites.

Figure 21 shows human KSR1 (hKSR1) sequences (hKSR1-KD-wt-CDS: SEQ ID NO. 43; hKSR1-KD-wt: SEQ ID NO. 44; hKSR1-KD-C922Y CDS: SEQ ID NO. 45; and hKSR1-KD-C922Y: SEQ ID NO. 46). The bolded residues indicate linker and restriction sites. Mutated residues are shaded in black.

Figure 22 shows human KSR1-KD-rlucII fusions cloned between KpnI and PmeI in pCDNA3.1-zeo (hKSR1-KD-wt-rlucII CDS: SEQ ID NO. 47; hKSR1-KD-wt-rlucII: SEQ ID NO. 48; hKSR1-KD-C922Y-rlucII CDS: SEQ ID NO. 49; and hKSR1-KD-C922Y-rlucII: SEQ ID NO. 50). The bolded residues indicate linker and restriction sites.

Figure 23 shows human MEK1 (hMEK1) sequences (hMEK1 CDS: SEQ ID NO. 51; and hMEK1: SEQ ID NO. 52).

Figure 24 shows human GFP10-MEK1 full length fusion cloned between NheI and XbaI in pCDNA3.1-zeo (GFP10-hMEK1 CDS: SEQ ID NO. 53; and GFP10-hMEK1: SEQ ID NO. 54). The bolded residues indicate linker and restriction sites.

Figure 25 shows the sequences of cloning oligonucleotides. Human BRAF (OL5_hBRAF_KD_+start_F: SEQ ID NO. 55; OL3_hBRAF_KD_+CAAX_+stop_R: SEQ ID NO. 56); human CRAF (OL5_hCRAF_KD_+start_F: SEQ ID NO. 57; OL5_hCRAF_KD_+CAAX_+stop_R: SEQ ID NO. 58); human KSR1 (OL5_hKSR1_KpnI_cloning_BRET: SEQ ID NO. 59; OL3_hKSR_XbaI_cloning_BRET: SEQ ID NO.60); and human MEK (OL5_hMEK1_KpnI_cloning_BRET: SEQ ID NO. 61;

OL3_hMEK_XbaI_cloning_BRET: SEQ ID NO. 62). The bolded residues indicate linker and restriction sites.

Figure 26 shows sequencing oligonucleotides (OL5_hBRAF_seq1_F: SEQ ID NO. 63; OL5_hBRAF_seq2_F: SEQ ID NO. 64; OL5_hCRAF_seq1_F: SEQ ID NO. 65; and
5 OL5_hCRAF_seq2_F: SEQ ID NO. 66)

Figure 27 shows mutagenesis oligonucleotides. The following primer pair were used to generate the side-to-side dimer interface mutant R481H in BRAF: OL5_hBRAF_R481H_F (SEQ ID NO. 67); and OL3_hBRAF_R481H_R (SEQ ID NO. 68). The following primer pair was used to introduce the C922Y hMEK1 interaction mutant in hKSR1: OL5_hKSR1_C922Y_F (SEQ ID NO. 69) and
10 OL3_hKSR1_C922Y_R (SEQ ID NO. 70). The bolded residues indicate linker and restriction sites.

DETAILED DESCRIPTION

In the following description of the embodiments, references to the accompanying Figures are by way of illustration of an example by which the embodiments described herein may be practiced. It will be understood that other embodiments may be made without departing from the scope of that
15 disclosed herein.

Definitions:

Unless otherwise specified, the following definitions apply throughout:

As used herein, the singular forms "a", "an", and "the" include plural referents unless the context clearly indicates otherwise. Thus, for example, reference to "a mutation" includes one or more of
20 such mutations and reference to "the method" includes reference to equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

As used herein, the term "comprising" is intended to mean that the list of elements following the word "comprising" are required or mandatory but that other elements are optional and may or may
25 not be present.

As used herein, the term "consisting of" is intended to mean including and limited to whatever follows the phrase "consisting of". Thus the phrase "consisting of" indicates that the listed elements are required or mandatory and that no other elements may be present.

As used herein, the term "RAF" is intended to refer to a protein, a polypeptide or fragment thereof,
30 encoded by a RAF gene. Examples of Wild-type (WT) human RAF proteins include the RAF protein isoforms known as A-RAF, B-RAF and C-RAF (e.g., genbank accession numbers P10398 for Homo sapiens A-RAF; P15056 for Homo sapiens B-RAF; and P04049 for Homo sapiens C-RAF). Examples of RAF xenologues are (e.g. genbank accession number P11346 for Drosophila

melanogaster pole hole (phl; RAF); P04627 for *Mus musculus* A-RAF; P28028 for *Mus musculus* B-RAF; and Q99N57 for *Mus musculus* C-RAF. Included in this definition are any functional RAF fragment, or any fusion of functional RAF fragments. Examples of these fragments include those that consist of, consist essentially of, or comprise the RAF kinase domain. Furthermore, the term

5 also encompasses any fusion of full length RAF, or a functional fragment thereof, with another polypeptide. These fusions include, but are not limited to, GST-RAF, HA tagged RAF, or Flag tagged RAF. These additional polypeptides may be linked to the N-terminus and/or C-terminus of RAF. Chimeric RAF protein, including a protein comprising a fusion of a RAF domain or domains with a portion of another protein, wherein the chimeric RAF retains the properties of human RAF,

10 are also included. Examples of chimeric RAF proteins include the fusion of any of the above RAF domains, or fragments thereof, to any domain or fragment of the following proteins such as, for example, GST, luciferase or GFP derivatives. RAF also includes any protein with at least 70% sequence identity with mammalian or non-mammalian RAF. The term also includes any conservative substitutions of amino-acid residues in RAF. The term "conservative substitution"

15 refers to replacement of an amino acid residue by a chemically similar residue, e.g., a hydrophobic residue for a separate hydrophobic residue, a charged residue for a separate charged residue, etc. Examples of conserved substitutions for non-polar R groups are alanine, valine, leucine, isoleucine, proline, methionine, phenylalanine, and tryptophan. Examples of substitutions for polar, but uncharged R groups are glycine, serine, threonine, cysteine, asparagine, or glutamine.

20 Examples of substitutions for negatively charged R groups are aspartic acid or glutamic acid. Examples of substitutions for positively charged R groups are lysine, arginine, or histidine. Furthermore, the term RAF includes conservative substitutions with non-natural amino-acids.

The following are Accession numbers for RAF cDNA and protein sequences for various species:

Accession numbers for RAF cDNA sequences

25 NM_080308 : *Drosophila melanogaster* pole hole (phl; RAF)
 NM_009703 : *Mus musculus* A-RAF
 NM_139294 : *Mus musculus* B-RAF
 AB057663: *Mus musculus* C-RAF
 X04790 : *Homo sapiens* A-RAF

30 NM_004333 : *Homo sapiens* B-RAF
 NM_002880 : *Homo sapiens* C-RAF

Accession numbers for RAF protein sequences

P11346 : *Drosophila melanogaster* pole hole (phl; RAF)

35 P04627 : *Mus musculus* A-RAF
 P28028 : *Mus musculus* B-RAF
 Q99N57 : *Mus musculus* C-RAF

P10398 : Homo sapiens A-RAF

P15056 : Homo sapiens B-RAF

P04049 : Homo sapiens C-RAF

5 As used herein, the terms "mutated RAF protein" and "mutated RAF polypeptide" are used interchangeably throughout and are intended to mean a WT RAF protein in which one or more amino acid residues have been changed. In certain examples described herein, the mutations include H449E, G450W, R481H, L487R, F488A, F488L, M489W, Y538F, A541E and K542E, which are located in the dimerization interface. Unless otherwise stated, amino acid residue
10 positions in RAF proteins refer to those of the *Drosophila melanogaster* sequences.

As used herein, the term "KSR" is intended to refer to a Kinase Suppressor of Ras protein, a polypeptide or fragment thereof, encoded by a KSR gene. Examples of Wild type (WT) human KSR proteins include the KSR protein isoforms known as KSR1 and KSR2 (e.g. genbank accession number A8MY87 for Homo sapiens kinase suppressor of ras 1 (KSR1) and Q6VAB6 :
15 for Homo sapiens kinase suppressor of ras 2 (KSR2). Examples of KSR xenologues are (e.g. genbank accession numbers Q24171 for *Drosophila melanogaster* kinase suppressor of ras (KSR); Q61097 for *Mus musculus* kinase suppressor of ras 1 (KSR1); and Q3UVC0 for *Mus musculus* kinase suppressor of ras 2 (KSR2). The term "KSR" also means any functional KSR fragment, or any fusion of functional KSR fragments. Examples of these fragments include those
20 that consist of, consist essentially of, or comprise the KSR kinase domain. Included in this definition are fusion of full length KSR, or a functional fragment thereof, with another polypeptide. These fusions include, but are not limited to, GST-KSR, HA tagged KSR, or Flag tagged KSR. These additional polypeptides may be linked to the N-terminus and/or C-terminus of KSR. Any chimeric KSR protein including a protein comprising a fusion of a KSR domain or domains with a
25 portion of another protein, wherein the chimeric KSR retains the properties of human KSR, are also included. Examples of chimeric KSR proteins include the fusion of any of the above KSR domains, or fragments thereof, to any domain or fragment of the following proteins such as, for example, GST, luciferase or GFP derivatives. KSR also includes any protein with at least 70% sequence identity with mammalian or non-mammalian KSR. The term also includes any conservative
30 substitutions of amino-acid residues in KSR. The term "conservative substitution" refers to replacement of an amino acid residue by a chemically similar residue, e.g., a hydrophobic residue for a separate hydrophobic residue, a charged residue for a separate charged residue, etc. Examples of conserved substitutions for non-polar R groups are alanine, valine, leucine, isoleucine, proline, methionine, phenylalanine, and tryptophan. Examples of substitutions for polar, but uncharged R groups are glycine, serine, threonine, cysteine, asparagine, or glutamine.
35 Examples of substitutions for negatively charged R groups are aspartic acid or glutamic acid.

Examples of substitutions for positively charged R groups are lysine, arginine, or histidine. Furthermore, the term KSR includes conservative substitutions with non-natural amino-acids.

The following are Accession numbers for KSR cDNA and protein sequences for various species:

Accession numbers for KSR cDNA sequences

- 5 NM_079512 : Drosophila melanogaster kinase suppressor of ras (KSR)
- NM_013571 : Mus musculus kinase suppressor of ras 1 (KSR1)
- DQ531035 : Mus musculus kinase suppressor of ras 2 (KSR2)
- NM_014238 : Homo sapiens kinase suppressor of ras 1 (KSR1)
- NM_173598 : Homo sapiens kinase suppressor of ras 2 (KSR2)

10

Accession numbers for KSR protein sequences

- Q24171 : Drosophila melanogaster kinase suppressor of ras (KSR)
- Q61097 : Mus musculus kinase suppressor of ras 1 (KSR1)
- Q3UVC0 : Mus musculus kinase suppressor of ras 2 (KSR2)
- 15 Q8IVT5: Homo sapiens kinase suppressor of ras 1 (KSR1)
- Q6VAB6 : Homo sapiens kinase suppressor of ras 2 (KSR2)

As used herein, the terms "mutated KSR protein" and "mutated KSR polypeptide" are used interchangeably throughout and are intended to mean a WT KSR protein in which one or more amino acid residues have been changed. In certain examples described herein, the mutations include H699E, G700W, R732H, L738R, F739A, F739L, M740W, Y790F, A793E and R794E, which are located in the dimerization interface. Unless otherwise stated, amino acid residue positions in KSR proteins refer to those of the Drosophila. melanogaster sequences.

As used herein, the term "mutation" is intended to mean any alteration in a gene which alters function or expression of the gene products, such as mRNA and the encoded for protein. This includes, but is not limited to, altering mutation, point mutation, truncation mutation, deletion mutation, frameshift mutation, and null mutation.

As used herein, the term "RAF gene" is intended to mean a gene encoding a RAF polypeptide having a dimerization interface. The RAF gene is a gene having about 50% or greater nucleotide sequence identity to at least one of human RAF isoforms (e.g. genbank accession numbers X04790 for Homo sapiens A-RAF; NM_004333 for Homo sapiens B-RAF; and NM_002880 for Homo sapiens C-RAF. Examples of RAF xenologues are (e.g. genbank accession numbers NM_080308 for Drosophila melanogaster pole hole (phl; RAF); NM_009703 for Mus musculus A-RAF; NM_139294 for Mus musculus B-RAF; and AB057663 for Mus musculus C-RAF)

As used herein, the term "KSR gene" is intended to mean a gene encoding a KSR polypeptide having a dimerization interface. The KSR gene is a gene having about 50% or greater nucleotide sequence identity to at least one of human KSR isoforms (e.g. genbank accession numbers NM_014238 for Homo sapiens kinase suppressor of ras 1 (KSR1); and NM_173598 for Homo sapiens kinase suppressor of ras 2 (KSR2)). Examples of KSR xenologues are (e.g. genbank accession numbers NM_079512 for Drosophila melanogaster kinase suppressor of ras (KSR); NM_013571 for Mus musculus kinase suppressor of ras 1 (KSR1); and DQ531035 for Mus musculus kinase suppressor of ras 2 (KSR2).

As used herein, the term "gene" refers to a nucleic acid comprising an open reading frame encoding a polypeptide, including both exon and (optionally) intron sequences. The nucleic acid may also optionally include non-coding sequences such as promoter or enhancer sequences. The term "intron" refers to a DNA sequence present in a given gene that is not translated into protein and is generally found between exons.

As used herein, the term "dimer interface" is intended to mean a site in the WT RAF or KSR polypeptide sequence or the mutated RAF or KSR polypeptide sequence, which reacts with a RAF or KSR substrate.

As used herein, the terms "RAF kinase domain" or "KSR kinase domain" are intended to mean the portion of the RAF or KSR proteins that are related in sequence to a generic protein kinase domain.

As used herein, the term "detectable label" is intended to mean a compound that may be linked to a RAF or KSR kinase domain, such that when the compound is associated with the domain, the label allows either direct or indirect recognition of the compound so that it may be detected, measured and quantified.

As used herein, the term "affinity tag" is intended to mean a ligand or group, which is linked to a RAF or KSR kinase domain to allow another compound to be extracted from a solution to which the ligand or group is attached.

As used herein, the term "nucleic acid" or a "nucleic acid molecule" as used herein refers to any DNA or RNA molecule, either single or double stranded and, if single stranded, the molecule of its complementary sequence in either linear or circular form. In discussing nucleic acid molecules, a sequence or structure of a particular nucleic acid molecule may be described herein according to the normal convention of providing the sequence in the 5' to 3' direction. With reference to nucleic acids described herein, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous in the naturally occurring genome of the organism in which it originated. For example, an "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such

as a plasmid or virus vector, or integrated into the genomic DNA of a prokaryotic or eukaryotic cell or host organism. Whenever applicable, the term "isolated nucleic acid" may also refer to a RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from other nucleic acids with which it would be associated in its natural state (i.e. in cells or tissues). An "isolated nucleic acid" (either DNA or RNA) may further represent a molecule produced directly by biological or synthetic means and separated from other components present during its production.

As used herein, the term "vector" is intended to mean a replicon, such as a plasmid, cosmid, bacmid, phage or virus, to which another genetic sequence or element (either DNA or RNA) may be attached so as to bring about the replication of the attached sequence or element.

As used herein, the terms "percent similarity", "percent identity" and "percent homology" when referring to a particular sequence are used as set forth in the University of Wisconsin GCG software program.

As used herein, the term "substantially pure" is intended to refer to a preparation comprising at least 50-60% by weight of a given material (e.g., nucleic acid, oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-95% by weight of the given compound. Purity is measured by methods appropriate for the given compound (e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like). Described herein are substantially pure mutated RAF or KSR isoforms (e.g., nucleic acids, oligonucleotides, proteins, fragments, mutants, etc.).

As used herein, the term "oligonucleotide" is intended to sequences, primers and probes as described herein, and is defined as a nucleic acid molecule comprised of two or more ribo- or deoxyribonucleotides, preferably more than three. The exact size of the oligonucleotide will depend on various factors and on the particular application and use of the oligonucleotide.

As used herein, the term "primer" is intended to refer to an oligonucleotide, either RNA or DNA, either single-stranded or double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed in the proper environment, is able to functionally act as an initiator of template-dependent nucleic acid synthesis. When presented with an appropriate nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme, suitable cofactors and conditions such as appropriate temperature and pH, the primer may be extended at its 3' terminus by the addition of nucleotides by the action of a polymerase or similar activity to yield a primer extension product. The primer may vary in length depending on the particular conditions and requirement of the application. For example, in diagnostic applications, the oligonucleotide primer is typically about 20-40, or more nucleotides in length. The primer must be of sufficient complementarity to the

desired template to prime the synthesis of the desired extension product, that is, to be able to anneal with the desired template strand in a manner sufficient to provide the 3' hydroxyl moiety of the primer in appropriate juxtaposition for use in the initiation of synthesis by a polymerase or similar enzyme. It is not required that the primer sequence represent an exact complement of the desired template. For example, a non-complementary nucleotide sequence may be attached to the 5' end of an otherwise complementary primer. Alternatively, non-complementary bases may be interspersed within the oligonucleotide primer sequence, provided that the primer sequence has sufficient complementarity with the sequence of the desired template strand to functionally provide a template-primer complex for the synthesis of the extension product.

As used herein, the term "probe" is intended to refer to an oligonucleotide, polynucleotide or nucleic acid, either RNA or DNA, whether occurring naturally as in a purified restriction enzyme digest or produced synthetically, which is capable of annealing with or specifically hybridizing to a nucleic acid with sequences complementary to the probe. A probe may be either single-stranded or double-stranded. The exact length of the probe will depend upon many factors, including temperature, source of probe and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide probe typically contains about 20-40 or more nucleotides in length, although it may contain fewer nucleotides. The probes herein are selected to be complementary to different strands of a particular target nucleic acid sequence. This means that the probes must be sufficiently complementary so as to be able to "specifically hybridize" or anneal with their respective target strands under a set of pre-determined conditions. Therefore, the probe sequence need not reflect the exact complementary sequence of the target. For example, a non-complementary nucleotide fragment may be attached to the 5' or 3' end of the probe, with the remainder of the probe sequence being complementary to the target strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the probe, provided that the probe sequence has sufficient complementarity with the sequence of the target nucleic acid to anneal therewith specifically.

With respect to single-stranded nucleic acids, particularly oligonucleotides, the term "specifically hybridizing" refers to the association between two single-stranded nucleotide molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA molecule as described herein, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence. Appropriate conditions enabling specific hybridization of single-stranded nucleic acid molecules of varying complementarity are well known in the art. For instance, one common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid

molecules of a specified sequence homology is set forth below (Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press):

$$T_m = 81.5\text{ }^{\circ}\text{C} + 16.6 \log [\text{Na}^+] + 0.41(\% \text{ G+C}) - 0.63 (\% \text{ formamide}) - 600/\#bp \text{ in duplex}$$

As an illustration of the above formula, using $[\text{Na}^+] = [0.368]$ and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1-1.5 with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C.

The stringency of the hybridization and wash depends primarily on the salt concentration and temperature of the solutions. In general, to maximize the rate of annealing of the probe with its target, the hybridization is usually carried out at salt and temperature conditions that are 20-25°C below the calculated T_m of the hybrid. Wash conditions should be as stringent as possible for the degree of identity of the probe for the target. In general, wash conditions are selected to be approximately 12-20°C below the T_m of the hybrid. With regard to the nucleic acids as described herein, a moderate stringency hybridization is defined as hybridization in 6xSSC, 5xDenhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C and washed in 2xSSC and 0.5% SDS at 55°C for 15 minutes. A high stringency hybridization is defined as hybridization in 6xSSC, 5xDenhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C, and washed in 1xSSC and 0.5% SDS at 65°C for 15 minutes. A very high stringency hybridization is defined as hybridization in 6xSSC, 5xDenhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C, and washed in 0.1xSSC and 0.5% SDS at 65°C for 15 minutes.

Alternatively, as used herein, the term "probe" is intended to mean a compound which is labeled with either a detectable label or an affinity tag, and which is capable of binding, either covalently or non-covalently, to a RAF or KSR kinase domain. When, for example, the probe is non-covalently bound, it may be displaced by a test compound. When, for example, the probe is bound covalently, it may be used to form cross-linked adducts, which may be quantified and inhibited by a test compound.

As used herein, the term "isolated protein" or "isolated and purified protein" is intended to refer to a protein produced by expression of an isolated nucleic acid molecule as described herein. Alternatively, this term may refer to a protein that has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in "substantially pure" form. "Isolated" is not meant to exclude artificial or synthetic mixtures with other compounds or materials, or the presence of impurities that do not interfere with the fundamental activity, and that may be present, for example, due to incomplete purification, or the addition of stabilizers.

As used herein, the term "amino acid" is intended to mean a radical derived from the corresponding α -amino acid by eliminating the hydroxyl of the carboxy group and one hydrogen of the α -amino group. For example, the terms Gln, Ala, Gly, Ile, Arg, Asp, Phe, Ser, Leu, Cys, Asn, and Tyr represent the residues of L-glutamine, L-alanine, glycine, L-isoleucine, L-arginine, L-aspartic acid, L-phenylalanine, L-serine, L-leucine, L-cysteine, L-asparagine, and L-tyrosine, respectively. Amino Acid residues are provided below:

Three and single letter abbreviations for α -amino acids used throughout are as follows:

Amino acid.	Abbreviation	Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Aspartic acid	Asp	D
Asparagine	Asn	N
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Isoleucine	Ile	I
Histidine	His	H
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

As used herein, the term "subject" is intended to mean humans and non-human mammals such as primates, cats, dogs, swine, cattle, sheep, goats, horses, rabbits, rats, mice and the like.

As used herein, the term "solid support" refers to any solid or stationary material to which reagents such as antibodies, antigens, and other test components can be attached. Examples of solid supports include, without limitation, microtiter plates (or dish), microscope (e.g. glass) slides, coverslips, beads, cell culture flasks, chips (for example, silica-based, glass, or gold chip), membranes, particles (typically solid; for example, agarose, sepharoseTM, polystyrene or magnetic beads), columns (or column materials), and test tubes. Typically, the solid supports are water insoluble.

As used herein, the term "instructional material" or a "user manual" includes a publication, a recording, a diagram, or any other medium of expression which can be used to communicate the usefulness of reagents for performing a method as described herein.

As used herein, the term "biological sample" is intended to refer to a subset of the tissues of a biological organism, its cells or component parts (e.g. body fluids, including but not limited to, blood, mucus, lymphatic fluid, synovial fluid, cerebrospinal fluid, saliva, amniotic fluid, amniotic cord blood, urine, vaginal fluid and semen).

5 We have discovered, using a combination of structural analysis, site-directed mutagenesis and functional studies *in vivo*, a dimerization interface in RAF and KSR. We have identified a number of residues within the RAF and KSR kinase domains which, when mutated, prevent the formation of oncogenic dimers. Through this, we have discovered that RAF catalytic function is regulated in response to a specific mode of dimerization of its kinase domain (which we term the side-to-side
10 dimer). Furthermore, we have discovered that the RAF-related pseudo-kinase KSR also participates in forming side-to-side heterodimers with RAF and thereby can trigger RAF activation. This mechanism provides an elegant explanation for the longstanding conundrum regarding RAF catalytic activation and provides an explanation for the capacity of KSR, despite lacking catalytic function, to directly mediate RAF activation. We have also demonstrated that RAF side-to-side
15 dimer formation is essential for aberrant signaling by oncogenic B-RAF mutants and we have identified an oncogenic mutation that acts specifically by promoting side-to-side dimer formation. These discoveries allow us to identify the side-to-side dimer interface of RAF as a potential therapeutic target for intervention in B-RAF-dependent tumourigenesis.

20 **I: Nucleic acid molecules, vectors, cells, transgenes and transgenic non-human mammals**

Described herein are mutated isoforms of RAF and KSR proteins. Furthermore, we have discovered that single point mutations in the dimerization interface of RAF or KSR kinase domains prevents the formation of side-to-side dimers, when compared to wild type RAF or KSR. The single point mutations in the RAF kinase domain are at residues H449, G450, R481H, L487, F488,
25 M489, Y538, A541 and K542 with the mutations being H449E, G450W, R481H, L487R, F488A, F488L, M489W, Y538F, A541E and K542E. The single point mutations in the KSR kinase domain are at residues H699, G700, R732, L738, F739, M740, Y790, A793 and R794 with the mutations being H699E, G700W, R732H, L738R, F739A, F739L, M740W, Y790F, A793E and R794E. We have also discovered that the isolated kinase domain of RAF forms homodimers in aqueous
30 solution. Similar behavior is expected for the isolated KSR kinase domain as well as heterodimers should form in aqueous solution upon mixing equimolar amounts of RAF and KSR kinase domains.

Thus, a substantially pure DNA molecule, such as genomic, cDNA, or a synthetic DNA molecule, encodes one of the mammalian or non-mammalian RAF or KSR isoforms in which one or more nucleotide substitutions has/have been incorporated into the dimerization interface.

35 In certain embodiments, DNA sequences are substantially identical to the DNA sequences, or a fragment thereof, as illustrated in Figures 14A through 14F (SEQ ID NOs 8, 10, 12, 14, 16, and

18). Another aspect features RNA, which is encoded by the DNA described herein. In one example, the RNA is mRNA. In another example, the RNA is antisense RNA.

Also contemplated are oligonucleotide probes, which specifically hybridize with the nucleic acid molecules as described herein. In certain examples, the probe specifically hybridizes with mutated
5 RAF or KSR nucleic acid molecules (e.g. a nucleic acid having a sequence encoding a mutated RAF or KSR protein) while not hybridizing with the wild type or "normal" sequence under high or very high stringency conditions. Primers capable of specifically amplifying mutated RAF or KSR encoding nucleic acids described herein are also contemplated herein. As mentioned previously, such oligonucleotides are useful as probes and primers for detecting, isolating or amplifying
10 mutated RAF or KSR genes.

Nucleic acid molecules encoding the mutated RAF or KSR proteins, as described herein, can be prepared by known general methods or isolated from appropriate biological sources using methods known in the art. Additionally, cDNA or genomic clones having homology with human and other known mammalian RAF or KSR, for example, mouse, rat, and the like, or non-mammalian RAF or
15 KSR, such as *Drosophila*, may be isolated from other species using oligonucleotide probes corresponding to predetermined sequences within the human RAF or KSR encoding nucleic acids.

Nucleic acids described herein may be maintained as DNA in any convenient vector. Accordingly, vectors comprising a nucleic acid molecule as described herein and more particularly a plasmid expression vector are encompassed. Also encompassed are host cells transformed with such
20 vectors and transgenic animals comprising such a nucleic acid molecule as described herein. Those cells and animals could serve as models of disease in order to study the mechanism of the function of the RAF or KSR gene and also allow for the screening of therapeutics.

In some embodiments, the vector, host cell or transgenic animal comprise a nucleic acid molecule (a transgene) encoding a mutated RAF or KSR protein that is expressed or delivered to tissues.
25 The host cell is a transformed and stable cell line constitutively expressing the mutant RAF or KSR isoform.

Methods for producing host cells and transgenic animals are known in the art. Host cells include, but are not limited to mammalian, yeast or bacterial cells. Transgenic animals can be selected from non-human mammals such as farm animals (such as pigs, goats, sheep, cows, horses, rabbits, and the like), rodents (such as rats, guinea pigs, mice, and the like), non-human primates (such as baboon, monkeys, chimpanzees, and the like), and domestic animals (such as dogs, cats, and the like) and wild and domestic (such as swans, ducks, fowl and the like). A transgenic animal is an animal having cells that contain a transgene which was introduced into the animal or an ancestor of the animal at a prenatal (embryonic) stage. The cells and transgenic animals can be useful to
30

identify mutated RAF or KSR proteins specific to each organ, and monitoring dimerization of the RAF and KSR in response to therapeutic treatment.

II: Mutated RAF or KSR polypeptides

A mutated RAF or KSR polypeptide sequence may have 80% homology or more with any of the amino acid sequences disclosed herein. A mutated RAF or KSR polypeptide sequence as described herein may also comprise at least 50 or more contiguous amino acids of any of sequences disclosed herein.

Mutated dimer interface residues in *Drosophila* RAF or *Drosophila* KSR and their equivalent positions in mammalian B-RAF or KSR1 are provided in the Tables below:

10

Dmel RAF (Acc. # P11346)	Hsap BRAF (Acc. # P15056)	Mmus BRAF (Acc. # P28028)
H449E	H477	H514
G450W	G478	G515
R481H	R509	R546
L487R	L515	L552
F488A	F516	F553
F488L	F516	F553
M489W	M517	M554
Y538F	Y566	Y603
A541E	A569	A606 ₁₅
K542E	K570	K607

Dmel KSR (Acc. # Q24171)	Hsap KSR1 (Acc. # Q8IVT5)	Mmus KSR1 (Acc. # Q61097)
H699E	H631	H583
G700W	G632	G584
R732H	R663	R615
L738R	L669	L621
F739A	F670	F622
F739L	F670	F622
M740W	M671	M623
Y790F	Y721	Y673
A793E	A724	A676
R794E	K725	K677

Other dimer interface residues in *Drosophila* RAF or *Drosophila* KSR and their equivalent positions in mammalian B-RAF or KSR1, and which are mutable include those in the following Tables:

Dmel RAF (Acc. # P11346)	Hsap BRAF (Acc. # P15056)	Mmus BRAF (Acc. # P28028) ⁰
E420	D448	D485
W422	W450	W487
W448	W476	W513
K478	R506	R543
K479	K507	K544
T480	T508	T545
H482	H510	H547
C483	V511	V548
Q502	Q530	Q567 ₂₅
D537	D565	D602
L560	L588	L625
S561	T589	T626
E687	E715	E752

Dmel KSR (Acc. # Q24171)	Hsap KSR1 (Acc. # Q8IVT5)	Mmus KSR1 (Acc. # Q61097)
K670	Q602	Q554
W672	W604	W556
W698	W630	W582
K729	R660	R612
N730	Q661	Q613
T731	T662	T614
H733	H664	H616
E734	E665	E617
S754	S685	S637
G789	G720	G672
K812	K743	K695
V813	V744	V696
E941	E876	E828

SwissProt accession numbers are provided for reference

30

Referring to Figures 14A through 14F, specifically SEQ ID NOs 7, 9, 11, 13, 15 and 17, the amino acid positions for the experimentally verified dimer interface residues are shaded in the protein sequences presented. In these Figures, predicted additional dimer interface residues are underlined.

In some embodiments, the mutated RAF or KSR polypeptide is an isolated mutated protein in which the mutations are located in the RAF or KSR kinase domain, specifically in the dimerization interface. In certain examples, the mutated RAF polypeptide comprises one or more mutations selected from H449E, G450W, R481H, L487R, F488A, F488L, M489W, Y538F, A541E and
5 K542E. In certain examples, the mutated KSR polypeptide comprises one or more mutations selected from H699E, G700W, R732H, L738R, F739A, F739L, M740W, Y790F, A793E and R794E.

Mutated RAF or KSR proteins or polypeptides as described herein may be prepared in a variety of ways, according to known methods. The proteins may be purified from appropriate sources, e.g.,
10 transformed bacterial or animal cultured cells or tissues, by immunoaffinity purification. The availability of nucleic acid molecules encoding mutated RAF or KSR protein enables production of the protein using *in vitro* expression methods and cell-free expression systems known in the art. *In vitro* transcription and translation systems are commercially available, e.g., from Promega or Invitrogen.

15 Alternatively, larger quantities of mutated RAF or KSR proteins or polypeptides may be produced by expression in a suitable prokaryotic or eukaryotic system. For example, part or all of a DNA molecule encoding for mutated RAF or KSR may be inserted into a plasmid vector adapted for expression in a bacterial cell, such as *E. coli*. Such vectors comprise the regulatory elements necessary for expression of the DNA in the host cell positioned in such a manner as to permit
20 expression of the DNA in the host cell. Such regulatory elements required for expression include promoter sequences, transcription initiation sequences and, optionally, enhancer sequences. Mutated RAF or KSR proteins or polypeptides produced by gene expression in a recombinant prokaryotic or eukaryotic system may be purified according to methods known in the art.

Thus, another embodiment includes a method of producing a mammalian mutated RAF or KSR
25 polypeptide includes providing a cell transformed with a nucleic acid sequence encoding a mammalian mutated RAF or KSR polypeptide positioned for expression in the cell. The mutated RAF or KSR polypeptide has an amino acid change at one of the positions depicted in Figures 14A through 14F (SEQ ID NOs 7, 9, 11, 13, 15 and 17) that correspond to specific dimerization interface residues. The transformed cell is cultured under conditions for expressing the nucleic
30 acid; which then produces the mammalian mutated RAF or KSR polypeptide.

A dominant-negative protein is a protein that antagonizes the action of its normal counterpart. A dominant-negative RAF would be a mutant RAF that prevents endogenous RAF from performing its natural (or oncogenic) function. Such a dominant-negative RAF (or KSR) protein could do so by sequestering away key proteins that normally act in concert with endogenous RAF (or KSR). For
35 example, overexpression of a kinase-defective RAF construct is known to act as a dominant-

negative in part by its ability to out-compete for endogenous RAS, which is normally critical for RAF activation.

Thus a dominant negative mutant polypeptide of mammalian RAF or KSR, wherein the mutant polypeptide comprises a kinase domain having a disabled dimerization interface and therefore
5 does not associate to a WT mammalian RAF or KSR dimerization interface.

The use of a dominant-negative polypeptide could be treating or preventing a disease in a subject, in which the disease being characterized by RAF/RAF homodimerization or RAF/KSR heterodimerization. This method comprises administering to the subject in need thereof, an expression vector encoding mutated RAF or KSR polypeptide, the mutated RAF or KSR
10 polypeptide being positioned in the vector for expression in a cell of the subject in which RAF/RAF homodimerization or RAF/KSR heterodimerization is taking place, so as to treat or prevent the disease.

III: Detection methods

Recombinant WT and mutated RAF or KSR polypeptides can be used during *in vitro* RAF or KSR
15 dimerization experiments to follow the dimerization of RAF or KSR protomers. RAF or KSR polypeptides mutants can also be co-transfected in mammalian cells with target protein substrates, such as WT RAF or KSR.

Changes in WT and mutated RAF or KSR polypeptide dimerization in response to a potential therapeutic agent, and across cell phenotypes, can be monitored by measuring the variation of the
20 levels of phosphorylated MEK in the presence or absence of rapamycin in animal cells such as S2 cells.

The RAF or KSR dimerization appears to be involved in many aspects of cancer from initiation to metastasis. One additional aspect includes a method of detecting in a subject susceptibility to express mutant RAF or KSR polypeptide. The method includes taking a biological sample from the
25 subject that contains a sufficient amount of a nucleic acid, for example, DNA, and sequencing predetermined regions of the DNA, which encodes a RAF or KSR mutated polypeptide. By comparing this sequence with a corresponding sequence from a non-susceptible control subject, a RAF or KSR mutation known to be indicative of the susceptibility can be identified.

Thus, a method of detecting the presence of a mutation in a RAF kinase domain or a KSR kinase
30 domain, comprises a) providing a WT RAF kinase domain or a WT KSR kinase domain and a suspected mutant RAF kinase domain or a mutant KSR kinase domain, each domain having a cysteine residue located at its N-terminus; b) incubating the WT RAF kinase domain or the WT KSR kinase domain and the suspected mutant RAF kinase domain or the suspected mutant KSR kinase domain with different cross-linking detectable labels; c) incubating together equimolar

amounts of the labelled WT RAF kinase domain or the labeled WT KSR kinase domain and detecting a signal from the detectable label so as to provide a dimerization reference signal; and d) incubating equimolar amounts of the labeled suspected mutant B-RAF kinase domain or the suspected mutant KSR kinase domain and detecting a signal from the detectable labels, an absent
 5 signal or a reduce signal compared to that of the dimerization reference signal being an indication that a mutant B-RAF kinase domain or a mutant KSR kinase domain is present.

Also included is a bioluminescence resonance energy transfer (BRET) fusion molecule, and method of use. The fusion molecule comprises three components: a bioluminescent donor protein (donor) and a fluorescent acceptor molecule (acceptor), wherein the acceptor can accept energy
 10 from the donor-generated luminescence when these components are in an appropriate spatial relationship and in the presence of an appropriate substrate. A modulator (a drug-like compound for example) can either influence the proximity/orientation of the donor and the acceptor and thereby the energy transfer between these components, or it can play a different role in affecting the energy transfer between the donor-generated activated product and the acceptor.

15 Thus, there is provided a method of monitoring the formation of RAF/RAF or RAF/KSR kinase domain dimers to detect mutations inhibiting dimerization or drug-like molecules interfering with dimerization. This method comprises a) fusing either (i) a RAF kinase domain or (ii) a KSR kinase domain at either of their N- or C-termini to a BRET donor or a BRET acceptor to produce donor
 20 labeled and acceptor labeled fusion proteins; b) expressing the fusion proteins to identify combinations that provide specific BRET signals; c) introducing dimer interface mutations into either of the labeled fusion proteins; d) expressing the labeled mutated fusion proteins with WT RAF or KSR kinase domains; e) measuring the BRET signals, a loss or significant reduction of the BRET signal using dimer interface mutations as opposed to mutations remote from the interface,
 25 being an indication that a specific BRET signal which depends on the RAF/RAF or RAF/KSR dimerization interface has been obtained.

In one example, the BRET donor is *renilla* luciferase variant II or rlucII and the BRET acceptor is GFP10. The acceptor label is Yellow Fluorescent Protein (YFP). The donor labeled fusion protein is SEQ ID NOs. 24, 34, 42 and 48, whereas the acceptor labeled fusion protein is SEQ ID NOs. 22,
 30 30, 40 and 54. The donor labeled mutated fusion proteins are SEQ ID NOs. 36, 50 and the acceptor labeled mutated fusion proteins are SEQ ID NO. 32.

IV: Antibodies and kits

Also provided are antibodies capable of immunospecifically binding to mutated RAF or KSR proteins and polypeptides as described herein. Such antibodies may include, but are not limited to,
 35 polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')₂ fragments, fragments produced by a Fab expression

library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Such antibodies may be may be used for immunoaffinity enrichment of the mutated RAF or KSR or they may be used in a kit for detecting in a subject the susceptibility to develop a condition or an increased likelihood of developing a condition characterized by dimerization of RAF and/or KSR.

- 5 Polyclonal antibodies directed toward mutated RAF or KSR protein, polypeptides or fragments thereof may be prepared according to standard methods. In one example, monoclonal antibodies are prepared, such that antibodies react immunospecifically with predetermined epitopes of the mutated RAF or KSR protein. In one example, the antibodies are immunologically specific to mutated RAF or KSR proteins and polypeptides. Monoclonal antibodies may be prepared
10 according to general methods known in the art. Polyclonal or monoclonal antibodies that immunospecifically interact with mutant RAF or KSR proteins can be utilized for identifying and purifying such proteins. For example, antibodies may be utilized for affinity separation of proteins with which they immunospecifically interact. Antibodies may also be used to immunoprecipitate proteins from a sample containing a mixture of proteins and other biological molecules.
- 15 One advantageous use of antibodies as described herein is in the use of a kit for monitoring the RAF or KSR dimerization activity of a cell or the binding of RAF or KSR to specific protein substrates such as the 14-3-3 proteins. This information may be used for purposes of diagnosis, prognosis or for predicting the response to treatment. Examples of diseases include cancer. The kit comprises a substantially pure antibody that specifically binds to a mammalian mutated RAF or
20 KSR polypeptide and a means for detecting the binding of the antibody to the mammalian RAF or KSR polypeptide.

V: Screening methods

- Because we have identified the amino acid residues involved in RAF/RAF homodimerization and RAF/KSR heterodimerization, we can use this knowledge to screen for potential therapeutic agents
25 which interact, either covalently or non-covalently, with the WT amino residue counterparts. Thus, one additional aspect includes methods of identifying biological agents or small molecules that modulate or prevent RAF or KSR dimerization activity in the cell or modification of the regulation of protein RAF or KSR dimerization. This could also be exploited for example to screen for inhibitors, activators or modulators of RAF or KSR dimerization. The identified agents or molecules could be
30 exploited as research reagents or for therapeutic purposes. The method could be used for in vitro screening assays using purified RAF or KSR WT polypeptides.

Generally speaking, there is provided a method of identifying inhibitors of RAF/RAF or RAF/KSR dimerization that bind to a RAF or KSR kinase domain, the RAF or KSR full protein or the kinase domain is bound to a support, and a potential inhibitor is added to the assay. Alternatively, the

potential inhibitor may be bound to the support and the RAF or KSR full protein or the kinase domain is added.

Additionally, the above described BRET assay can be used as a method of identifying a potential inhibitor of RAF/RAF homodimerization. This method comprises a) fusing a RAF kinase domain at either of its N- or C-termini to a BRET donor or a BRET acceptor to produce donor labeled and acceptor labeled fusion proteins; b) expressing the fusion proteins to identify combinations that provide specific BRET signals; c) introducing dimer interface mutations into either of the labeled fusion proteins; d) expressing the labeled mutated fusion proteins with WT RAF kinase domains; e) contacting the interface with the potential inhibitor; and f) measuring the BRET signals, a loss or significant reduction of the BRET signal for the wild-type RAF/RAF BRET pair being an indication that the inhibitor is specifically bound to the interface.

There are a number of ways in which to determine the binding of a potential inhibitor to the RAF or KSR kinase domain. In one way, the potential inhibitor, for example, may be fluorescently or radioactively labeled and binding determined directly. For example, this may be done by attaching the RAF or KSR full protein or the kinase domain to a solid support, adding a detectably labeled potential inhibitor, washing off excess reagent, and determining whether the amount of the detectable label is present on the solid support. Numerous blocking and washing steps may be used, which are known to those skilled in the art.

In some cases, only one of the components is labeled. For example, specific residues, such as those identified as described herein, in the RAF or KSR kinase domain may be labeled. Alternatively, more than one component may be labeled with different labels; for example, using I¹²⁵ for the RAF or KSR domain, and a fluorescent label for the potential inhibitor.

As used herein, the terms "drug candidate", "test compounds" or "potential inhibitor" are used interchangeably and describe any molecule, for example, protein, oligopeptide, small organic molecule, polysaccharide, polynucleotide, and the like, to be tested for bioactivity. The compounds may be capable of inhibiting the formation of RAF/RAF homodimers or RAF/KSR heterodimers.

Drug candidates can include various chemical classes, although typically they are small organic molecules having a molecular weight of more than 100 and less than about 2,500 Daltons. Candidate agents typically include functional groups necessary for structural interaction with proteins, for example, hydrogen bonding and lipophilic binding, and typically include at least an amine, carbonyl, hydroxyl, ether, or carboxyl group. The drug candidates often include cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more functional groups.

Drug candidates can be obtained from any number of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed

synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional
5 chemical, physical and biochemical means.

Competitive screening assays may be done by combining a RAF or KSR kinase domain and a labeled probe to form a probe:RAF or KSR kinase domain complex in a first sample followed by adding a potential inhibitor from a second sample. The binding of the potential inhibitor is determined, and a change or difference in binding between the two samples indicates the
10 presence of a test compound capable of binding to the RAF or KSR kinase domain and potentially modulating the RAF or KSR's dimerizing ability.

In one case, the binding of the potential inhibitor is determined through the use of competitive binding assays. In this embodiment, the probe is labeled with a fluorescent label. Under certain circumstances, there may be competitive binding between the potential inhibitor and the probe.
15 Potential inhibitors which displace the probe, resulting in a change in fluorescence as compared to control, are considered to bind to the RAF or KSR kinase domain.

In one case, the potential inhibitor may be labeled. The potential inhibitor is added first to the RAF or KSR domain for a time sufficient to allow binding to form a complex.

Formation of the probe:RAF or KSR domain complex typically require incubations of between 4°C and 40°C, for between 10 minutes to about 1 hour to allow for high-throughput screening. Any
20 excess of reagents are generally removed or washed away. The potential inhibitor is then added, and the presence or absence of the labeled component is followed, to indicate binding to the RAF or KSR kinase domain.

In one case, the probe is added first, followed by the potential inhibitor. Displacement of the probe
25 is an indication the potential inhibitor is binding to the RAF or KSR domain and thus is capable of binding to, and potentially modulating or inhibiting the dimerization of RAF and KSR. Either component can be labeled. For example, the presence of probe in the wash solution indicates displacement by the potential inhibitor. Alternatively, if the potential inhibitor is labeled, the presence of the probe on the support indicates displacement.

30 In one case, the potential inhibitor may be added first, with incubation and washing, followed by the probe. The absence of binding by the probe may indicate the potential inhibitor is bound to the RAF or KSR domain with a higher affinity. Thus, if the probe is detected on the support, coupled with a lack of potential inhibitor binding, may indicate the potential inhibitor is capable of binding to the RAF or KSR kinase domain.

Modulation is tested by screening for a potential inhibitor's ability to modulate the activity of RAF or KSR and includes combining a potential inhibitor with a RAF or KSR kinase domain, as described above, and determining an alteration in the biological activity of RAF or KSR. Therefore in this case, the potential inhibitor should both bind to the RAF or KSR kinase domain (although this may
 5 not be necessary), and alter its biological activity as defined herein.

Positive controls and negative controls may be used in the assays. All control and test samples are performed multiple times to obtain statistically significant results. Following incubation, all samples are washed free of non-specifically bound material and the amount of bound probe determined. For example, where a radiolabel is employed, the samples may be counted in a scintillation
 10 counter to determine the amount of bound potential inhibitor.

Typically, the signals that are detected in the assay may include fluorescence, resonance energy transfer, time resolved fluorescence, radioactivity, fluorescence polarization, plasma resonance, or chemiluminescence and the like, depending on the nature of the label. Detectable labels useful in performing screening assays as described herein include a fluorescent label such as Fluorescein,
 15 Oregon green, dansyl, rhodamine, tetramethyl rhodamine, texas red, Eu³⁺; a chemiluminescent label such as luciferase; calorimetric labels; enzymatic markers; or radioisotopes such as tritium, I¹²⁵ and the like

Affinity tags, which may be useful in performing the screening assays as described herein include biotin, polyhistidine and the like.

20 **EXAMPLES**

1. S2 Expression Plasmids

Copper-inducible pMet vectors were used for functional assays conducted in S2 cells as previously described (Douziech, M., Sahmi, M., Laberge, G. & Therrien, M. A KSR/CNK complex mediated by HYP, a novel SAM domain-containing protein, regulates RAS-dependent RAF activation in
 25 *Drosophila*. *Genes Dev* 20, 807-19 (2006)), Roy, F., Laberge, G., Douziech, M., Ferland-McCollough, D. & Therrien, M. KSR is a scaffold required for activation of the ERK/MAPK module. *Genes Dev* 16, 427-38 (2002)). The FRB-RAF^{K455S} fusion construct was assembled by inserting an *Ase1/Not1* PCR fragment encompassing residues 328-738 of RAF into the *Ase1/Not1* site of FRB-KSR (Roy, F., Laberge, G., Douziech, M., Ferland-McCollough, D. & Therrien, M. KSR is a scaffold
 30 required for activation of the ERK/MAPK module. *Genes Dev* 16, 427-38 (2002)). The KSR-RAF chimera-A corresponds to KSR¹⁻⁶⁶⁵ fused to RAF⁴¹⁷⁻⁷³⁹, whereas chimera-B replaced the N-lobe of KSR (a.a. positions 666-757) with the one of RAF (a.a. positions 417-505). In both cases, the RAF N-lobe contained a K455M change to catalytically impair its kinase activity and thereby mimicked kinase-inert KSR. Variant full length *Drosophila* KSR, RAF or FRB/FKBP fusion mutants were

generated by QuickChange™ mutagenesis (Stratagene). Mutagenized cDNAs were fully sequenced to verify that only the desired mutations had been introduced.

2. S2 Cell Assays

S2 cells were maintained in serum-free insect cell medium (Sigma) at 27°C. Cells were seeded at a density of 1.75×10^6 cells/ml 24h prior to transfection. Between 10 to 300 ng (or up to 900 ng for KSR_R732H) of DNA was transfected per construct using Effectene (Qiagen). dsRNAs were produced and used in RNAi experiments as described (Roy, F., Laberge, G., Douziech, M., Ferland-McCollough, D. & Therrien, M. KSR is a scaffold required for activation of the ERK/MAPK module. *Genes Dev* 16, 427-38 (2002)). Protein expression was induced by adding CuSO₄ (0.7 mM) 36h before harvesting the cells. For FRB/FKBP-mediated dimer formation, rapamycin (Sigma) was added (1 μM) to the medium 2h prior to harvesting the cells. Lysates, immunoprecipitations, western blot procedures and antibodies were essentially as previously described (Douziech, M., Sahmi, M., Laberge, G. & Therrien, M. A, KSR/CNK complex mediated by HYP, a novel SAM domain-containing protein, regulates RAS-dependent RAF activation in *Drosophila*, *Genes Dev*, 20, 807-19 (2006)).

3. Bacterial Protein Expression and Purification

B-RAF (residues 448-723) and mutant series (R481H, L487R and L487R/E558K) were recombinantly expressed from pProEx (Invitrogen) plasmid in *E. coli* BL21 cells as TEV protease-cleavable 6xHis-tagged fusions. To increase the level of soluble protein expression in *E. coli*, 16 specific mutations (remote from the side-to-side dimer interface) were introduced in B-RAF as described Tsai, J. et al., Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity, *Proc Natl Acad Sci, U S A*, 105, 3041-6 (2008). Expressed proteins were bound to Ni-NTA and eluted with imidazole and subjected to TEV protease treatment. Further purification was performed by subtractive Ni-NTA and size exclusion (Superdex™ 200) chromatography.

4. Homology Modeling

A multiple sequence alignment of KSR and RAF kinase domains was used to build a structural model of the kinase domain of *Drosophila* KSR (residues 670-945) in SWISS-MODEL (Schwede, T., Kopp, J., Guex, N. & Peitsch, M. C. SWISS-MODEL: An automated protein homology-modeling server. *Nucleic Acids Res* 31, 3381-5 (2003)). An initial model with a total energy of -7474.3 KJ/mol was generated using the structure of the kinase domain of B-RAF as a template (chain A of PDB entry 1UWH) (Wan, P. T. et al., Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF, *Cell*, 116, 855-67 (2004)). This model was manually edited in COOT (Emsley, P. & Cowtan, K., Coot: model-building tools for molecular graphics, *Acta Crystallogr D Biol Crystallogr*, 60, 2126-32 (2004)) and a poorly modelled loop spanning residues

821-838 was removed. To generate the KSR/RAF side-to-side heterodimer, the modelled structure of KSR was superimposed onto chain A of PDB entry 1UWH.

5. Analytical Ultracentrifugation

Equilibrium sedimentation was performed with a Beckman Optima™ XL-A ultracentrifuge and An60Ti rotor. B-RAF samples were prepared in 20 mM Tris (pH 7.5), 200 mM NaCl, 5% glycerol and 1.5 mM TCEP for analysis. Data was collected at 4°C for three protein concentrations (25 µM, 12.5 µM, and 6.25 µM) at three rotor speeds (13,000 rpm, 18,000 rpm and 23,000 rpm for B-RAF_wt and B-RAF_R481H or 12,000 rpm, 17,000 rpm and 25,000 rpm for B-RAF_L487R and B-RAF-L487R/E558K). Model analysis of the data was performed simultaneously in a global curve-fitting procedure (Origin software, Beckman). For this, data collected at 13,000 rpm and 18,000 rpm for B-RAF_wt was analyzed at all three protein concentrations; data collected at 18,000 rpm and 23,000 rpm for B-RAF_R481H was analyzed at all three protein concentrations; data collected at 17,000 rpm and 25,000 rpm for B-RAF_L487R was analyzed at all three protein concentrations; data collected at 17,000 rpm and 25,000 rpm for B-RAF_L487R/E558K at 25 µM and 12.5 µM was analyzed. The term “global” refers to fits across all rotor speeds for a given concentration.

The global self association fit yielded an average molecular weight (MW) of 57,978 Da for B-RAF_wt. The ratio of the observed average MW to the theoretical MW of the monomer is 1.9:1 suggesting that the sample contains mostly dimers. A single-species dimer model (shown by the red line; Figure 3C) best fit the observed data (blue circles; Figure 3C), indicated by the random distribution of the residuals – a measure of goodness of fit (the residual is the difference between the observed value and the predicted value). For B-RAF_R481H, the global self association fit yielded an average MW of 34,544 Da. The ratio of the observed average MW to the theoretical MW of the monomer is 1.1:1 suggesting that the sample contains mostly monomers. A single-species monomer model (red line; Figure 3C) best fit the observed data (blue circles; Figure 3C). For B-RAF_L487R, the global self association fit yielded an average MW of 48,636 Da. The ratio of the observed average MW to the theoretical MW of the monomer is 1.5:1 suggesting that the sample contains a mixture of monomers and dimers. Consistent with this, a monomer-dimer model (red line; Figure 12) resulted in the best fit to the observed data (blue circles; Figure 12) with a dissociation constant (Kd) of 2 µM (Note: In order to reliably estimate Kd values from an AUC experiment, both species in a monomer-dimer equilibrium need to be sufficiently represented in solution; in our AUC analyses, we observed such a monomer-dimer equilibrium only for the B-RAF_L487R dimer mutant). For B-RAF_L487R/E558K, the global self association fit yielded an average MW of 55,472 Da. The ratio of the observed average MW to the theoretical MW of the monomer is 1.8:1 suggesting that the sample contains mostly dimers and the data (blue circles; Figure 12) was best fit to a single-species dimer model (red line; Figure 12).

6. RNA preparation and Quantitative real-time PCR (qPCR)

S2 cells were treated with specific RNAi for four days and total RNA was extracted using Trizol™ reagent (Invitrogen) according to the manufacturer's instructions. qPCR analyses were performed as follows. A total of 2 µg of RNA was reverse transcribed using the High Capacity cDNA Archive Kit with random primers (Applied Biosystems, Foster City, CA) as described by the manufacturer. Primer and probe sets from Universal ProbeLibrary were used for quantitative real-time PCR. Primers were chosen so that the amplified regions did not overlap with the areas targeted by dsRNAs used for RNAi. PCR reactions for 384-well plate formats were performed using 2 µl of cDNA, 5 µl of the TaqMan™ fast Universal PCR Master Mix (Applied Biosystems, CA), 2 µM of each primer and 1 µM of the Universal TaqMan probe in a total volume of 10 µl. The ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) was used to detect the amplification level. The relative quantification of target genes was determined by using the CT method. Briefly, the Ct (threshold cycle) values of target genes were normalized to an endogenous control gene (Rpl49) ($CT = Ct_{\text{target}} - Ct_{\text{Rpl49}}$) and compared with a calibrator (wild type): $CT = Ct_{\text{Sample}} - Ct_{\text{Calibrator}}$. Relative expression (RQ) or fold change was calculated using the Sequence Detection System (SDS) 2.2.2 software (Applied Biosystems) and the formula $RQ = 2^{-CT}$.

dsRNA primers:

GFP amplicon

top 5'- CGTAAACGGCCACAAGTTCAG (SEQ ID NO: 71)
 20 bottom 5'- ACGAACTCCAGCAGGACCATG (SEQ ID NO: 72)

RAS amplicon

top 5'- AATACAACTGGTCGTCGTTG (SEQ ID NO: 73)
 bottom 5'- AATCTACGATTCGGCTTGTTT (SEQ ID NO: 74)

25

CNK amplicon

top 5'- TTTGGACAGATCTATATGCAG (SEQ ID NO: 75)
 bottom 5'- TCGGTTCAAAGGTCTCCAG (SEQ ID NO: 76)

HYP amplicon

top 5'- CCGATTGTGTCACCCCTAAT (SEQ ID NO: 77)
 bottom 5'- CCACTTGAGCACATCGCTAA (SEQ ID NO: 78)

CK2α amplicon

top 5'- GACACTTCCTAGTGCGGCTCGCGTG (SEQ ID NO: 79)

bottom 5'- GTAATCATACATCTGGTAATCTACC (SEQ ID NO: 80)

14-3-3ε amplicon

5 top 5'- TGA CTGAGCGCGAGAACAATG (SEQ ID NO: 81)

bottom 5'- TCTTCTGCCTGCATATCGGAC (SEQ ID NO: 82)

14-3-3ζ amplicon

top 5'- GACAGTCGATAAGGAAGAGCTGG (SEQ ID NO: 83)

bottom 5'- TCGTTCAGTGTGTCCAGCTC (SEQ ID NO: 84)

10

7. Screening assays

Two independent assays were developed to monitor RAF dimerization. The first is a FRET (fluorescence resonance energy transfer) assay. It is based on the observation that bacterially-expressed human B-RAF kinase domain form dimers in solution.

15 The second assay exploits the BRET (bioluminescence resonance energy transfer) technology to assess for RAF homodimerization or RAF-KSR heterodimerization using a cell-based system.

A. FRET assay

A single cysteine residue is engineered at the N-terminus of the wild-type or mutant B-RAF kinase domain for cross-linking fluorescent probes. Following bacterial expression and purification, independent
20 batch of proteins are labeled either with Alexa 555 (donor) or Alexa 647 (acceptor). Labeled proteins are re-purified and then combined in equimolar ratios. FRET detection is carried out using a Luminescence Spectrometer. Various controls are conducted in parallel. For instance, no FRET signal is detected when labeled proteins are tested alone. Similarly, no FRET signal or a significantly reduced one is detected when dimer interface mutants (e.g. R481H-like) are tested in combination with wild-type B-RAF.

25 B. BRET assay

The BRET assay can use either BRET1 or BRET2 as a means of measuring BRET signals. We used BRET2 donor (*renilla* luciferase variant II or rlucII) and acceptor (GFP10) fusions rather than BRET1 fusions (rluc and YFP) as well as the addition of a CAAX-box to target RAF to the plasma membrane, since the BRET2 system is more sensitive and has a higher signal to noise ratio (Kocan, See et al., 2008, *J Biomol Screen.* 13(9): 888-98), to independently fuse RAF and KSR kinase domains at either their N- or
30 C-terminus. The addition of the CAAX-box is frequently used to generate BRAF gain of function alleles and is reported in the literature (Leevers, Paterson et al., 1994, *Nature.* 369(6479): 411-4). We focused on

the human BRAF kinase domain (BRAF-KD) expressed from the pCDNA3.1 plasmid backbone (Invitrogen) in a HEK293 transfection setup.

The assay included co-transfection of rlucII fused to the N-terminus of the human BRAF-KD (referred to below as the donor) and of N-terminally GFP10-tagged hBRAF-KD (referred to below as the acceptor) both targeted to the plasma membrane with a CAAX-box. Transfections were performed in HEK293T cells with PEI as a transfection reagent in a 6-well format with varying molar ratios of pCDNA3.1 donor:acceptor constructs (0:1, 0.25:1, 0.5:1, 1:1, 2:1, 5:1, 10:1 and 20:1). 48 hours post transfection, the cells were washed and resuspended in tyrode buffer and transferred to opaque microtiter plates. GFP10 raw signal was read on a FlexStation 3 plate reader (Molecular Devices) and BRET signals were read using a Mithras LB 940 plate reader following the addition of DeepBlue™ C (DBC) at a concentration of 10µM. The data was then analysed using the GraphPad Prism software package.

The assay was highly reproducible and the BRET ratio obtained for the rlucII-BRAF-KD-CAAX versus GFP10-BRAF-KD-CAAX pair at saturating concentration of the acceptor construct was consistently between 4.7 and 4.9.

We also generated the dimer interface mutation R481H of the BRAF-KD and measured its impact on the affinity of the BRAF-BRAF interaction. The BRAF wt-wt pair yielded a significantly higher BRET ratio than when the R481H mutant was introduced as the donor or acceptor construct (Figure 15). This reduction was reproducible and significant in terms of both the BRET_{max} and BRET₅₀ ratios (Figure 15). This was indicative of a significant decrease in BRAF-BRAF affinity when the dimer interface is perturbed.

Altogether, the BRET assay is specific for the BRAF-KD vs BRAF-KD interaction and is highly sensitive to the genetic alteration of the now well-characterized dimer interface (Hatzivassiliou, Song et al. 2010, *Nature* Mar 18;464(7287): 431-5; Poulikakos, Zhang et al. 2010, *Nature* Mar 18;464(7287): 427-30; Rajakulendran, Sahmi et al. 2009, *Nature* Sep 24;461(7263): 542-5).

All BRET assays were developed with the human B-RAF (hBRAF), C-RAF (hCRAF), KSR1 (hKSR1) and MEK1 (hMEK1) isoforms (see Figures 16 through 27 and SEQ ID NO's: 19 through 70). In Figures 16 through 27, CDS stands for coding sequences. And KD stands for kinase domain.

The mutagenised residues are labeled according to their position in the *Drosophila* orthologous protein sequence. Thus, the R481H mutation of *Drosophila* RAF corresponds to the R509H mutation of human B-RAF, and the C922Y mutation of *Drosophila* KSR corresponds to the C722Y mutation of human KSR1.

Results and discussion

In *Drosophila*, RAF activation is regulated by a core complex that notably includes the proteins RAS, CNK, HYP and KSR amongst others (Claperon, A. & Therrien, M. KSR and CNK: two scaffolds regulating RAS-mediated RAF activation. *Oncogene* 26, 3143-58 (2007)). Of these proteins, the function of KSR (Kinase Suppressor of Ras) in RAF activation remains controversial. KSR contains a kinase domain of closest sequence similarity to RAF (Manning, G., Whyte, D. B., Martinez, R., Hunter, T. & Sudarsanam, S. The protein kinase complement of the human genome. *Science* 298, 1912-34 (2002)) and was initially thought to drive RAF activation by virtue of its kinase activity. However, subsequent studies have been inconclusive in demonstrating this point and thus relegated KSR as a pseudo-kinase (Boudeau, J., Miranda-Saavedra, D., Barton, G. J. & Alessi, D. R. Emerging roles of pseudokinases. *Trends Cell Biol* 16, 443-52 (2006)). Because of its capacity to bring MEK to RAF, the function of KSR is currently considered to be that of an organizing centre (or scaffold) in the ERK pathway (Kolch, W. Coordinating ERK/MAPK signalling through scaffolds and inhibitors. *Nat Rev Mol Cell Biol* 6, 827-37 (2005)).

We previously showed in *Drosophila* S2 cells that co-overexpression of KSR with RAF and MEK stimulated RAF-dependent MEK phosphorylation (Douziech, M., Sahmi, M., Laberge, G. & Therrien, M. A KSR/CNK complex mediated by HYP, a novel SAM domain-containing protein, regulates RAS-dependent RAF activation in *Drosophila*. *Genes Dev* 20, 807-19 (2006)). If KSR was solely acting as a scaffold, we reasoned that overexpression of KSR without co-overexpression of its scaffold partners would perturb the optimal stoichiometry of KSR containing complexes with the net effect of decreasing RAF activation. Since we observed increased RAF activation, this suggested that KSR might possess an inherent RAF activating capacity that becomes apparent upon overexpression. To investigate whether KSR can stimulate increasing RAF activation in a concentration dependent manner (as would be the case if KSR possessed an intrinsic RAF activating capacity), we titrated in increasing amounts of KSR in S2 cells and monitored the effect by assessing RAF-dependent MEK phosphorylation (Roy, F., Laberge, G., Douziech, M., Ferland-McCollough, D. & Therrien, M. KSR is a scaffold required for activation of the ERK/MAPK module. *Genes Dev* 16, 427-38 (2002)). As shown in Figure 1A, increasing levels of KSR correspondingly increased MEK phosphorylation. Surprisingly, this KSR-dependent RAF activation was unperturbed by RNAi-mediated knockdown of RAS (Figure 1A; see Figure 13A for a demonstration of the activity and specificity of the RAS dsRNA). Moreover, co-overexpression of a constitutively active RAS (RAS^{V12}) under these conditions did not considerably augment MEK phosphorylation, suggesting that KSR can drive RAF activation independently of RAS activity when overexpressed in S2 cells (Figure 1A). These results suggest a role for KSR in RAF activation beyond a scaffold-only function.

To more rigorously rule out a scaffolding function as the origin of the observed stimulatory effect of overexpressed KSR on RAF, we used RNAi to knockdown a subset of known scaffold partners of KSR. Interestingly, in this context RAF activation was also unperturbed by RNAi-mediated knockdown of CNK or HYP (also known as AVE), which are normally required under physiological conditions (Douziech, M., Sahmi, M., Laberge, G. & Therrien, M. A KSR/CNK complex mediated by HYP, a novel SAM domain-containing protein, regulates RAS-dependent RAF activation in *Drosophila*. *Genes Dev* 20, 807-19 (2006)), Douziech, M. et al. Bimodal regulation of RAF by CNK in *Drosophila*. *Embo J* 22, 5068-78 (2003)) and Roignant, J. Y., Hamel, S., Janody, F. & Treisman, J. E. The novel SAM domain protein Aveugle is required for Raf activation in the *Drosophila* EGF receptor signaling pathway. *Genes Dev* 20, 795-806 (2006) (Figure 1B). Studies with mammalian cells recently suggested that CK2 (bound to KSR1) phosphorylates and activates B-RAF/C-RAF (Ritt, D. A. et al. CK2 Is a component of the KSR1 scaffold complex that contributes to Raf kinase activation. *Curr Biol* 17, 179-84 (2007)). We found that KSR not only potently stimulated RAF activation in the presence of RNAi-mediated knockdown of CK2 (Figure 1C), but that mutating the proposed CK2 binding site on KSR or the sites of CK2-mediated phosphorylation on RAF (Ritt, D. A. et al. CK2 Is a component of the KSR1 scaffold complex that contributes to Raf kinase activation. *Curr Biol* 17, 179-84 (2007)) had no impact on the ability of KSR to drive RAF activation under our overexpression conditions (Figure 1C). Taken together, these results suggest that the overexpression of KSR in S2 cells unmasks an inherent activation potential on RAF beyond its well established role as a scaffold.

We previously showed that the capacity of KSR to bind MEK was required for the ability of RAF to phosphorylate MEK (Roy, F., Laberge, G., Douziech, M., Ferland-McCollough, D. & Therrien, M. KSR is a scaffold required for activation of the ERK/MAPK module. *Genes Dev* 16, 427-38 (2002)). This result supported a scaffolding role for KSR in RAF activation. More recently, we identified a mutation in KSR (R732H) within its kinase domain that completely abolished its RAF activating capacity yet fully retained its ability to bind MEK and RAF Douziech, M., Sahmi, M., Laberge, G. & Therrien, M. A KSR/CNK complex mediated by HYP, a novel SAM domain-containing protein, regulates RAS-dependent RAF activation in *Drosophila*. *Genes Dev* 20, 807-19 (2006)) (Figure 2). This mutant provided a starting point for unraveling the mechanism by which KSR directly activates the catalytic function of RAF.

Since the kinase domain of KSR is most similar to that of RAF (Manning, G., Whyte, D. B., Martinez, R., Hunter, T. & Sudarsanam, S. The protein kinase complement of the human genome. *Science* 298, 1912-34 (2002)), we hypothesized that the previously determined crystal structure of the kinase domain of human B-RAF (the human orthologue of *Drosophila* RAF) might provide a good model to discern the mechanism of action of the KSR_R732H mutation. Indeed, Arg732 is not only invariant across all KSR proteins, it is invariant across the larger RAF/KSR family (but not in other closely related kinases; Figure 4). Intriguingly, while the structure of the kinase domain of

B-RAF was reported as a monomer (Wan, P. T. et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell* 116, 855-67 (2004)), the asymmetric unit of the crystal in fact contains two RAF kinase domains that interact in a unique side-to-side fashion involving the N-lobe of their kinase domains (Figure 6A). This mode of dimerization, which was not appreciated to date, was observed in a total of five subsequent RAF structure analyses (Wan, P. T. et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell* 116, 855-67 (2004)), (Hansen, J. D. et al. Potent and selective pyrazole-based inhibitors of B-Raf kinase. *Bioorg Med Chem Lett* 18, 4692-5 (2008)) (in distinct crystal lattices), suggesting that the mode of dimerization/oligomerization is functionally relevant rather than an artifact of crystal packing (Figure 6B). Side-to-side dimerization of the RAF kinase domain buries a large surface area ($\sim 1280 \text{ \AA}^2$) and provocatively involves helix αC , a key structural element whose conformation serves a regulatory function in numerous protein kinases (Huse, M. & Kuriyan, J. The conformational plasticity of protein kinases. *Cell* 109, 275-82 (2002)) (Figure 6A). Most notably, a specific mode of dimerization involving helix αC underlies an allosteric mechanism for kinase activation for both PKR (Dar, A. C., Dever, T. E. & Sicheri, F. Higher-order substrate recognition of eIF2 α by the RNA-dependent protein kinase PKR. *Cell* 122, 887-900 (2005)) and EGFR (Zhang, X., Gureasko, J., Shen, K., Cole, P. A. & Kuriyan, J. An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. *Cell* 125, 1137-49 (2006)) kinase domains (Figure 6C). As the structure of the RAF kinase domain adopts a productive conformation in the dimeric crystal configuration, we reasoned that side-to-side dimerization itself might directly modulate the attainment of an active kinase conformation of RAF.

Projection of KSR/RAF conserved residues onto the RAF crystal structure revealed that nearly the entire side-to-side dimer contact surface of RAF, but no other surfaces, are conserved across the larger KSR/RAF family (Figure 3A; Figure 4). This suggested that KSR might form an analogous dimer structure. Moreover, the position of Arg481 (the equivalent of Arg732 in KSR; Figure 4) at the center of the side-to-side dimer interface of the B-RAF crystal structure (Figure 3B) hinted at the basis by which the mutation of Arg732 in KSR might exert a functional effect by perturbing dimerization (for simplicity, we used the *Drosophila* RAF numbering scheme for discussion of human B-RAF positions; see the Table below for list of residue equivalence between B-RAF and *Drosophila* RAF).

<i>Drosophila</i> RAF	Human B-RAF
Trp422	Trp450
Trp448	Trp476
His449	His477
Gly450	Gly478
Lys478	Arg506
Lys479	Lys507
Thr480	Thr508
Arg481	Arg509
His482	His510

<i>Drosophila</i> RAF	Human B-RAF
Cys483	Val511
Leu487	Leu515
Phe488	Phe516
Met489	Met517
Gln502	Gln530
Asp537	Asp565
Tyr538	Tyr566
Ala541	Ala569
Lys542	Lys570
Glu558	Glu586
Leu560	Leu588
Ser561	Thr589
Glu687	Glu715

In order to investigate the potential of the RAF kinase domain to form dimers in solution, we performed analytical ultracentrifugation experiments (Figure 3C). Equilibrium sedimentation analysis confirmed that RAF can form dimers under the conditions tested (i.e. micromolar concentrations). Consistent with the mode of dimerization seen in the crystal structure, mutation of Arg481 in B-RAF converted it to a predominant monomer in solution. This result shows that the side-to-side dimer configuration of RAF visualized in the crystal environments is also sampled in solution. Based on these findings, we reasoned that the R732H mutation in KSR most likely perturbs KSR's ability to form an analogous side-to-side homodimer or to form a side-to-side heterodimer with RAF. This in turn could explain the mechanism by which the KSR_R732H mutation abolishes RAF activation.

If KSR mediates RAF activation by a mechanism involving the formation of a specific side-to-side homodimer with itself (i.e. KSR/KSR side-to-side homodimer) or a heterodimer with the kinase domain of RAF (i.e. KSR/RAF side-to-side heterodimer), then mutation of other dimer interface residues on KSR in close vicinity to Arg732 might also impair RAF activation. Using our minimal KSR/RAF/MEK co-overexpression activation assay, we found this to be the case. Specifically, individual mutation of four additional residues (G700W, F739A, M740W and Y790F) on KSR severely impeded its ability to induce RAF activation (Figure 5A). If KSR mediates RAF activation by forming a specific side-to-side heterodimer with the kinase domain of RAF, then mutations of the corresponding positions (residues) on RAF should also impair RAF activation. As shown in Figure 5A, we also found this to be the case. In contrast, control mutations remote from the side-to-side dimer interface on the kinase domains of both KSR and RAF showed no significant effect on RAF activation (Figure 5A). We note that none of the dimer interface mutations in KSR detectably affected the KSR/MEK interaction, indicating that the mutations did not simply destroy protein fold (data not shown). These results confirm that the integrity of the side-to-side dimer interface on KSR and on RAF is essential for RAF activation.

While our results above are consistent with the possibility that KSR and RAF heterodimerize through their kinase domains, it is equally possible that KSR/KSR side-to-side homodimers might instead contribute to RAF activation. To demonstrate that the formation of side-to-side kinase domain heterodimers by KSR and RAF *per se* leads to RAF activation, we employed the
5 FRB/FKBP fusion protein system to inducibly promote KSR/RAF side-to-side heterodimer formation by the addition of rapamycin *in vivo* (Muthuswamy, S. K., Gilman, M. & Brugge, J. S. Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers. Mol Cell Biol 19, 6845-57 (1999)).

Towards this end, we fused a region encompassing the minimal kinase domains of KSR and RAF
10 to the FRB and FKBP fragments, respectively (Roy, F., Laberge, G., Douziech, M., Ferland-McCollough, D. & Therrien, M. KSR is a scaffold required for activation of the ERK/MAPK module. Genes Dev 16, 427-38 (2002)) (See Figure 5B for schematic). The use of the FRB/FKBP fusion in conjunction with a myristoylation signal on the FKBP fusion construct (to localize it to the membrane) allowed us to tightly modulate heterodimerization of the kinase domains in a
15 rapamycin-dependent manner (Roy, F., Laberge, G., Douziech, M., Ferland-McCollough, D. & Therrien, M. KSR is a scaffold required for activation of the ERK/MAPK module. Genes Dev 16, 427-38 (2002)). In this setup, we observed that promoting the KSR/RAF heterodimer by addition of rapamycin was indeed sufficient to potently activate RAF as evidenced by the elevated levels of phosphorylated MEK (Figure 5B). RAF activation was selectively perturbed by ten specific
20 mutations at the side-to-side dimer interface on both KSR (H699E, G700W, R732H, L738R, F739A, F739L, M740W, Y790F, A793E and R794E) and on RAF (H449E, G450W, R481H, L487R, F488A, F488L, M489W, Y538F, A541E and K542E), but not by control mutations outside the side-to-side dimer interface of KSR or RAF (Figure 5B; Figure 8). Taken together, these results indicate that formation of the side-to-side heterodimer between KSR and RAF kinase domains is both
25 sufficient and necessary for RAF activation under the conditions tested.

As both RAF and KSR likely form identical side-to-side dimers, by virtue of having near identical dimerization surfaces (Figure 3A), it is conceivable that both KSR/RAF heterodimers and RAF/RAF homodimers might equally promote RAF activation, assuming RAF activation and downstream signaling is solely dependent on forming the kinase domain side-to-side dimer. To investigate
30 whether the RAF/RAF homodimers can also lead to RAF activation, we used the FRB/FKBP/rapamycin system to drive side-to-side homodimer formation of RAF kinase domains *in vivo* (see Figure 5C for schematic). To ensure our interpretation of side-to-side dimer formation induced activation is not confounded by trans autophosphorylation activity within the RAF/RAF homodimer, we introduced a mutation (K455S) in the FRB-RAF fusion to catalytically impair its
35 kinase activity (i.e. to effectively mimic the kinase dead state of KSR). As shown in Figure 5C, rapamycin induced formation of RAF/RAF homodimers can indeed drive RAF activation in a manner dependent on the ability to form the side-to-side dimers (Figure 5C).

Although RAF/RAF homodimers are competent for activation, the level of activation is not as robust as that resulting from KSR/RAF heterodimers (based on quantification of induced MEK phosphorylation levels in the presence and absence of rapamycin; not shown). If the side-to-side dimer surfaces are in fact functionally equivalent on both KSR and RAF, this observation suggests that the KSR kinase domain may have a second function that is not shared with RAF. Based on the fact that KSR can stably bind MEK while RAF cannot (Roy, F., Laberge, G., Douziech, M., Ferland-McCollough, D. & Therrien, M. KSR is a scaffold required for activation of the ERK/MAPK module. *Genes Dev* 16, 427-38 (2002)), we reasoned that this may be the root of the difference. Since the side-to-side dimerization surface is comprised mainly by the N-lobe of KSR and RAF kinase domains, and MEK binding function is critically dependent on the C-lobe of KSR (Roy, F., Laberge, G., Douziech, M., Ferland-McCollough, D. & Therrien, M. KSR is a scaffold required for activation of the ERK/MAPK module. *Genes Dev* 16, 427-38 (2002)), then a RAF N-lobe-KSR C-lobe chimera might possess both essential functions of the KSR kinase domain. If true, one would predict that substitution of the N-lobe of RAF into KSR, but not the whole kinase domain of RAF into KSR, would lead to the maintenance of KSR's ability to promote RAF mediated phosphorylation of MEK. This indeed proved to be the case. As shown in Figure 7, overexpression of a form of KSR with a full kinase domain swap with RAF (Figure 7A, *Chimera-A*) poorly activated RAF, while overexpression of a form with just an N-lobe swap (Figure 7A, *Chimera-B*) was as potent as wild type KSR in promoting MEK phosphorylation by RAF (Figure 7B). Confirming that MEK binding is indeed constrained to the C-lobe of KSR, *Chimera-B* but not *Chimera-A* bound to MEK as assessed by co-immunoprecipitation (Figure 7B). Taken together, these results highlight two distinct functions for the kinase domain of KSR in RAF signaling. Firstly, the kinase domain of KSR functions as a scaffold whereby it binds to MEK and recruits it to RAF (i.e. KSR mediates RAF substrate targeting). Secondly, the kinase domain of KSR forms a side-to-side heterodimer with the kinase domain of RAF that underlies an allosteric mechanism for RAF catalytic activation.

Recent studies with mammalian cells, where multiple RAF isoforms exist, have found that RAF activation can also occur upon the physical juxtaposition of two isoforms of RAF mediated by 14-3-3 proteins (Weber, C. K., Slusky, J. R., Kalmes, H. A. & Rapp, U. R. Active Ras induces heterodimerization of cRaf and BRaf. *Cancer Res* 61, 3595-8 (2001)), (Rushworth, L. K., Hindley, A. D., O'Neill, E. & Kolch, W. Regulation and role of Raf-1/B-Raf heterodimerization. *Mol Cell Biol* 26, 2262-72 (2006)). Intriguingly, this activation route is independent of a phospho-transfer mechanism as reflected by the fact that in such RAF/RAF heterodimers, a kinase-dead isoform of RAF can activate a wild-type isoform of RAF (Chen, C., Lewis, R. E. & White, M. A. IMP modulates KSR1-dependent multivalent complex formation to specify ERK1/2 pathway activation and response thresholds. *J Biol Chem* 283, 12789-96 (2008)). This behaviour is highly reminiscent of how KSR activates RAF. We reasoned that 14-3-3 proteins, which are intrinsically dimeric, act to promote the specific side-to-side dimer conformation we see in the RAF crystal structure in a manner analogous to our forced FRB-RAF/FKBP-RAF system (Figure 5C). Consistent with this

possibility, our modeling studies showed that the binding of dimeric 14-3-3 proteins concurrently to the C-terminal extension of two RAF kinase domains is fully compatible with the adoption of a side-to-side dimer configuration (Figure 10).

5 Interestingly, the 14-3-3 consensus binding site in human RAF is conserved in both RAF and KSR molecules in fly and in other organisms (Figure 9A), suggesting that 14-3-3 could also act to promote RAF homodimers and more potent KSR/RAF heterodimers in flies. Demonstrating that 14-3-3 is indeed relevant for RAF activation in flies, we found that depletion of endogenous 14-3-3 proteins perturbed KSR-dependent RAF activation (Figure 9B). Consistent with the notion that 14-3-3 mediates dimerization of KSR with RAF, mutation of the consensus 14-3-3 site in both KSR
10 and RAF impaired RAF activation (Figure 9B). These results suggest that 14-3-3 proteins might act to promote specific KSR/RAF and RAF/RAF side-to-side kinase domain dimers.

Together, our study indicates that dimerization of the RAF kinase domain with KSR or with other RAF molecules is central to its activation mechanism. We posit that other regulatory events that impinge on RAF activation may also act by modulating dimerization. In this regard, the large group
15 of scaffolding proteins that act together with RAF and KSR, such as 14-3-3 proteins, may serve to spatially and temporally regulate the formation of side-to-side dimers (Douziech, M., Sahmi, M., Laberge, G. & Therrien, M. A KSR/CNK complex mediated by HYP, a novel SAM domain-containing protein, regulates RAS-dependent RAF activation in *Drosophila*. *Genes Dev* 20, 807-19 (2006)), Garnett, M. J., Rana, S., Paterson, H., Barford, D. & Marais, R. Wild-type and mutant B-
20 RAF activate C-RAF through distinct mechanisms involving heterodimerization. *Mol Cell* 20, 963-9 (2005)), Rushworth, L. K., Hindley, A. D., O'Neill, E. & Kolch, W. Regulation and role of Raf-1/B-Raf heterodimerization. *Mol Cell Biol* 26, 2262-72 (2006)), Chen, C., Lewis, R. E. & White, M. A. IMP modulates KSR1-dependent multivalent complex formation to specify ERK1/2 pathway activation and response thresholds. *J Biol Chem* 283, 12789-96 (2008)). Moreover, the fact that
25 the formation of B-/C-RAF heterodimers appears to depend on RAS activity (Garnett, M. J., Rana, S., Paterson, H., Barford, D. & Marais, R. Wild-type and mutant B-RAF activate C-RAF through distinct mechanisms involving heterodimerization. *Mol Cell* 20, 963-9 (2005). strongly suggests that RAS may also play a role in forming side-to-side kinase domain dimers. In the absence of RTK/RAS activation, a regulatory element in the N-terminus of RAF engages the C-terminal kinase
30 domain to inhibit catalytic activity by an unknown mechanism (Chong, H. & Guan, K. L. Regulation of Raf through phosphorylation and N terminus-C terminus interaction. *J Biol Chem* 278, 36269-76 (2003)). We reason that this autoinhibitory interaction may interfere with the ability of the kinase domain to adopt a productive dimer configuration.

Although dependent on many more components, the activation mechanism of RAF appears
35 analogous in principle, if not execution, to those employed by the PKR and EGFR protein kinases. In the case of the eIF2 γ protein kinase PKR, the attainment of a specific dimer configuration by the kinase domain is regulated by the binding of dsRNA viral by-products to regions N-terminal to the

kinase domain (Dar, A. C., Dever, T. E. & Sicheri, F. Higher-order substrate recognition of eIF2alpha by the RNA-dependent protein kinase PKR. *Cell* 122, 887-900 (2005)), Dey, M. et al. Mechanistic link between PKR dimerization, autophosphorylation, and eIF2alpha substrate recognition. *Cell* 122, 901-13 (2005). In the case of EGFR kinase, adoption of a unique dimer/oligomer configuration by its kinase domain is regulated by the binding of growth factors to the extracellular ligand binding domain of the receptors (Zhang, X., Gureasko, J., Shen, K., Cole, P. A. & Kuriyan, J. An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. *Cell* 125, 1137-49 (2006)) (Figure 6C). Reflecting the importance of self interaction in the function of all three protein kinase families, residues comprising the self interaction surfaces of the kinase domain in addition to the catalytic infrastructure are evolutionarily conserved within each kinase family. In this regard, KSR is essentially equivalent to a RAF molecule. In effect, we reason that RAF and KSR evolved from a single ancestral progenitor, one that possessed both protein kinase catalytic activity and stable substrate (MEK) binding function. Following a gene duplication event (Claperon, A. & Therrien, M. KSR and CNK: two scaffolds regulating RAS-mediated RAF activation. *Oncogene* 26, 3143-58 (2007)), one gene dispensed with phospho-transfer function (i.e. KSR) and the other dispensed with the ability to stably bind MEK substrate (i.e. RAF). However, both maintained the ability to form allosteric dimers and this selective pressure maintained the side-to-side dimer interface and interdependence between KSR and RAF proteins in ERK signaling.

The mapping of human cancer causing mutations to the activation segment of B-RAF proved unequivocally that the activation segment of RAF is also a key modulator of its catalytic function (Wan, P. T. et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell* 116, 855-67 (2004)), (Davies, H. et al. Mutations of the BRAF gene in human cancer. *Nature* 417, 949-54 (2002)). Consistent with this, we previously found that a mutation in the activation segment of *Drosophila* RAF (RAF-AL^{ED}) strongly hyperactivated its catalytic activity (Douziech, M., Sahmi, M., Laberge, G. & Therrien, M. A KSR/CNK complex mediated by HYP, a novel SAM domain-containing protein, regulates RAS-dependent RAF activation in *Drosophila*. *Genes Dev* 20, 807-19 (2006)), suggesting that it likely acts via a similar mechanism as those identified in human cancers (Wan, P. T. et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell* 116, 855-67 (2004)). This raises the question of how kinase domain dimerization and the modulation of activation segment conformation are coordinated. Both events may be essential for the transmission of a downstream signal or each event may be sufficient on its own. If both are essential, then oncogenic activation segment mutants of RAF should still be sensitive to dimer interface mutations. Suggesting that this in fact is the case, introduction of a mutation (R481H) within the side-to-side dimer interface in *Drosophila* RAF effectively nullifies the aberrant signaling properties of RAF-AL^{ED} (Figure 11A).

Intriguingly, while most oncogenic RAF mutations act through modulation of the activation segment, one particular mutation, RAF_E558K (E586K in human B-RAF), is located on the opposite surface of the kinase domain from the activation segment (Wan, P. T. et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell 116, 855-67 (2004)) and its mechanism of kinase activation remained enigmatic. Most conspicuously, Glu558 lies on the side-to-side dimer interface (Figure 11B). If dimerization is indeed critical for RAF activation, we questioned whether RAF_E558K might promote kinase activity by promoting dimerization. We reasoned that mutation of Glu558 to the longer Lys (E558K) could potentially introduce a hydrogen bond with Ser561 (conservative Thr589 in B-RAF) on the second RAF protomer thereby promoting dimer formation (Figure 11B). Indeed, as tested below we found that the RAF_E558K mutation promoted kinase domain dimerization in solution. Wild type RAF kinase domain is predominantly a dimer in solution (at the micromolar concentrations tested), which prevented a direct test of the RAF_E558K mutant for enhanced dimerization potential (Figure 3C). To circumvent this problem, we employed the RAF_L487R dimer mutant which displayed a weak monomer-dimer binding equilibrium in solution (Figure 12).

Thus, introduction of the E558K mutation (RAF_L487R/E558K double mutant) transitioned RAF_L487R back to a predominantly dimeric state (Figure 6). To investigate how the RAF_E558K mutation functions to hyperactivate RAF *in vivo*, we used the FRB/FKBP/rapamycin system to assess RAF activation in S2 cells. When the E558K mutation was introduced in the kinase-dead (K455S) background (FRB-RAF_K455S/E558K double mutant), it displayed no activity when tested alone (not shown), but strongly hyperactivated the FKBP-RAF counterpart in a rapamycin dependent manner (Figure 11C). Taken together, the ability of the E558K mutant to act *in trans* (i.e. in the context of a kinase dead mutant) *in vivo*, and the ability of the E558K mutation to promote kinase domain dimerization *in vitro* strongly suggests that the mechanism by which the oncogenic RAF_E558K mutation acts is by promoting side-to-side dimers. Given these results, it is now possible to develop small molecules strategies that are directed at preventing the formation of side-to-side dimers by RAF and which can serve as a therapeutic for RAF-dependent human tumors, one that would complement conventional strategies currently directed at inhibiting RAF enzymatic activity by blocking the catalytic cleft (Wu, S., Guo, W. & Fang, B. Development of small-molecule inhibitors of raf. Recent Patents Anti-Infect Drug Disc 1, 241-6 (2006)).

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the present discovery and scope of the appended claims.

CLAIMS

1. A biosensor for monitoring the dimerization of human BRAF by Bioluminescence Resonance Energy Transfer (BRET), the biosensor comprising:
an isolated human embryonic kidney (HEK) cell expressing:
 - (a) a first fusion molecule comprising the amino acid sequence of SEQ ID NO: 34; and
 - (b) a second fusion molecule comprising the amino acid sequence of SEQ ID NO: 30.
2. The biosensor of claim 1, wherein said HEK cell is an HEK293T cell line.
3. A method for determining whether a test agent increases or decreases the homodimerization of human BRAF, the method comprising:
 - a) providing the biosensor of claim 1 or 2 in the presence or absence of said test agent; and;
 - b) measuring the fluorescence signal emitted by said second fusion molecule in said cell,
wherein a higher fluorescence signal measured in the presence of the test agent is indicative that said test agent increases the homodimerization of human BRAF, and a lower fluorescence signal measured in the presence of the test agent is indicative that said test agent inhibits the homodimerization of human BRAF.
4. The method of claim 3, wherein said method further comprises contacting the cell with a substrate for the donor bioluminescent protein present in said first fusion molecule.
5. The method of claim 3 or 4, wherein said substrate is Coelenterazine 400a (DeepBlue C™).
6. The method of any one of claims 3 to 5, further comprising:
 - (c) measuring the bioluminescent signal emitted by said first fusion molecule,
and

(d) determining the ratio [fluorescent acceptor signal/bioluminescence donor signal];

wherein a higher ratio measured in the presence of the test agent is indicative that said test agent increases the homodimerization of human BRAF, and a lower ratio measured in the presence of the test agent is indicative that said test agent inhibits the homodimerization of human BRAF.

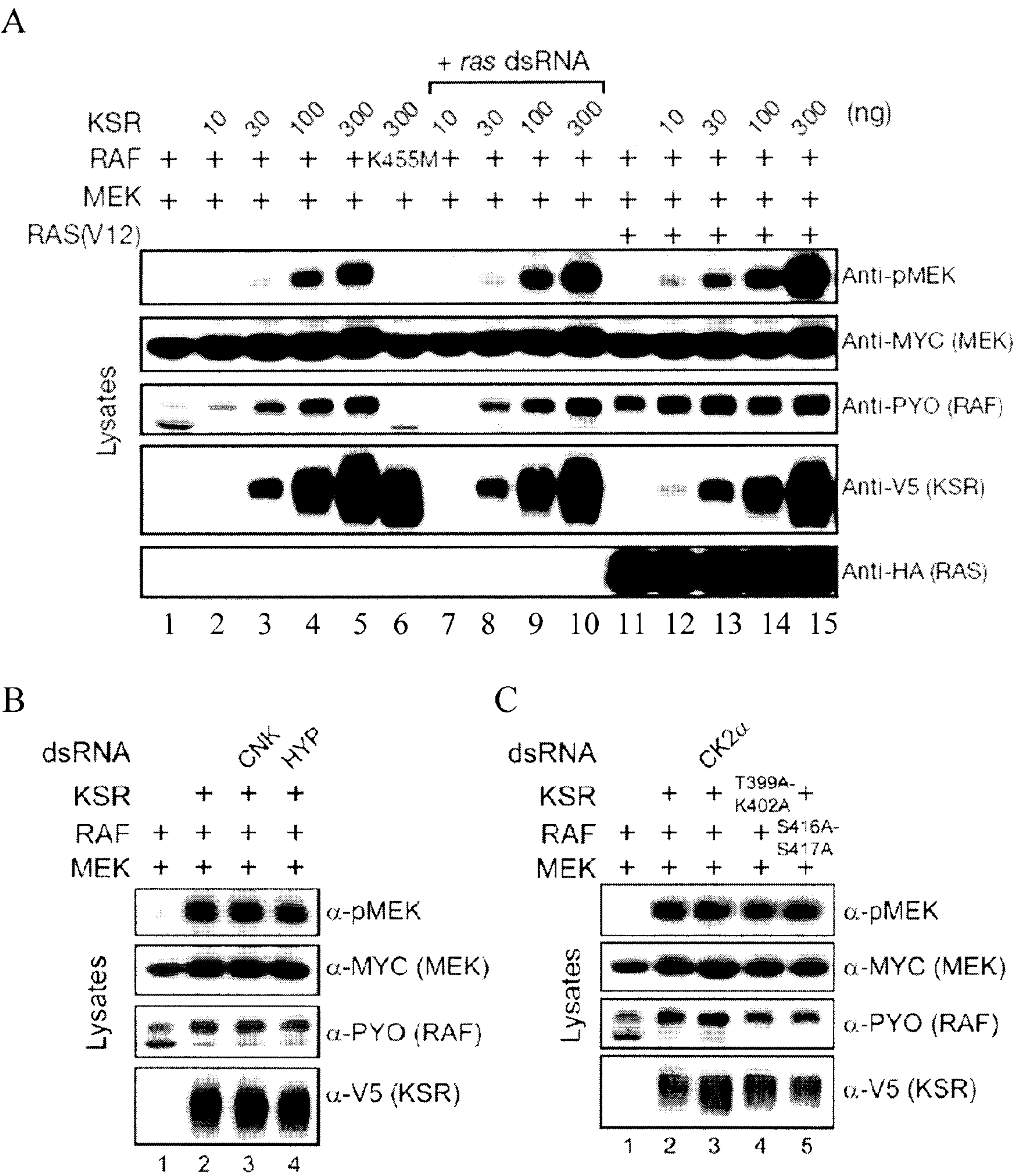


Figure 1

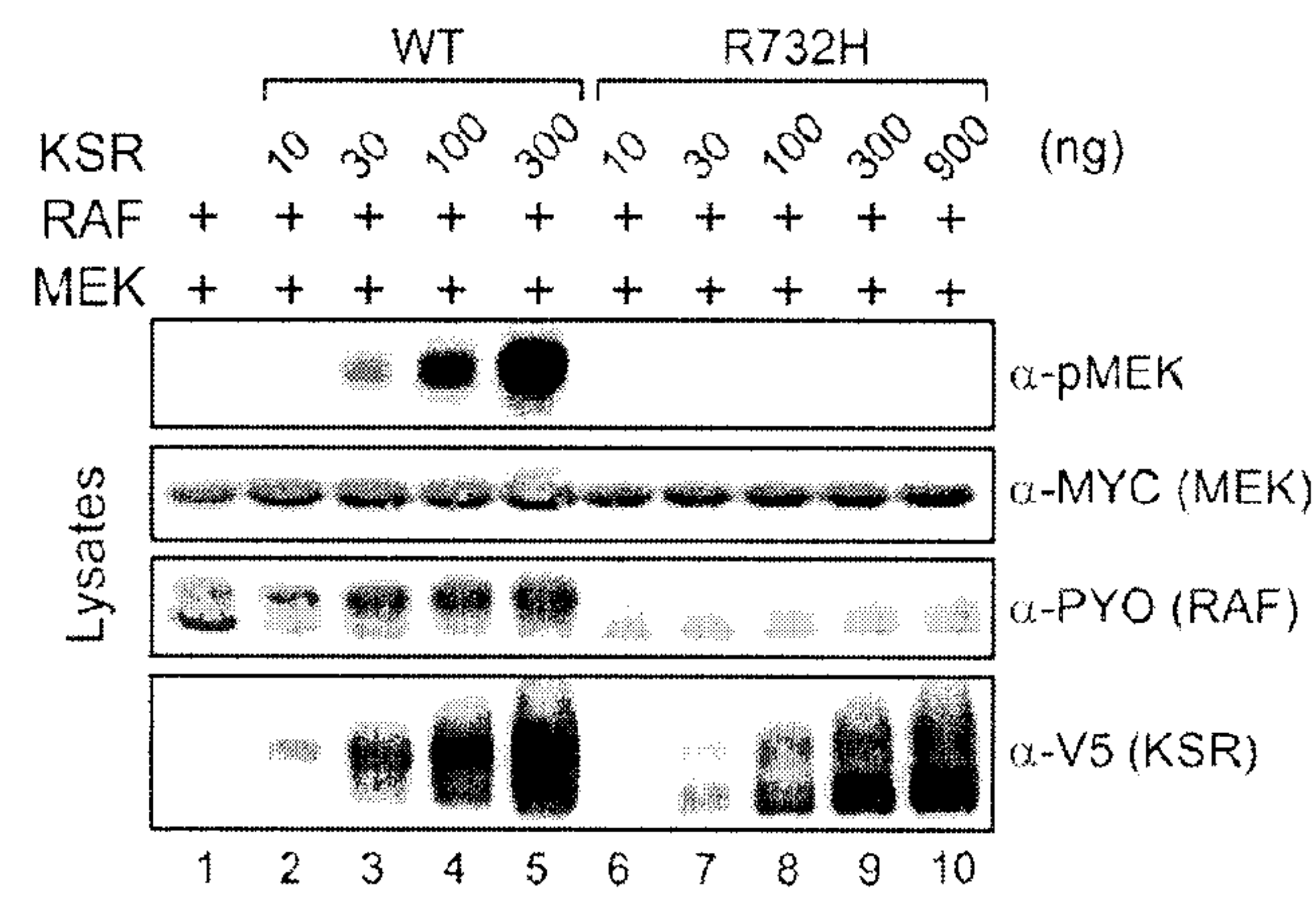


Figure 2

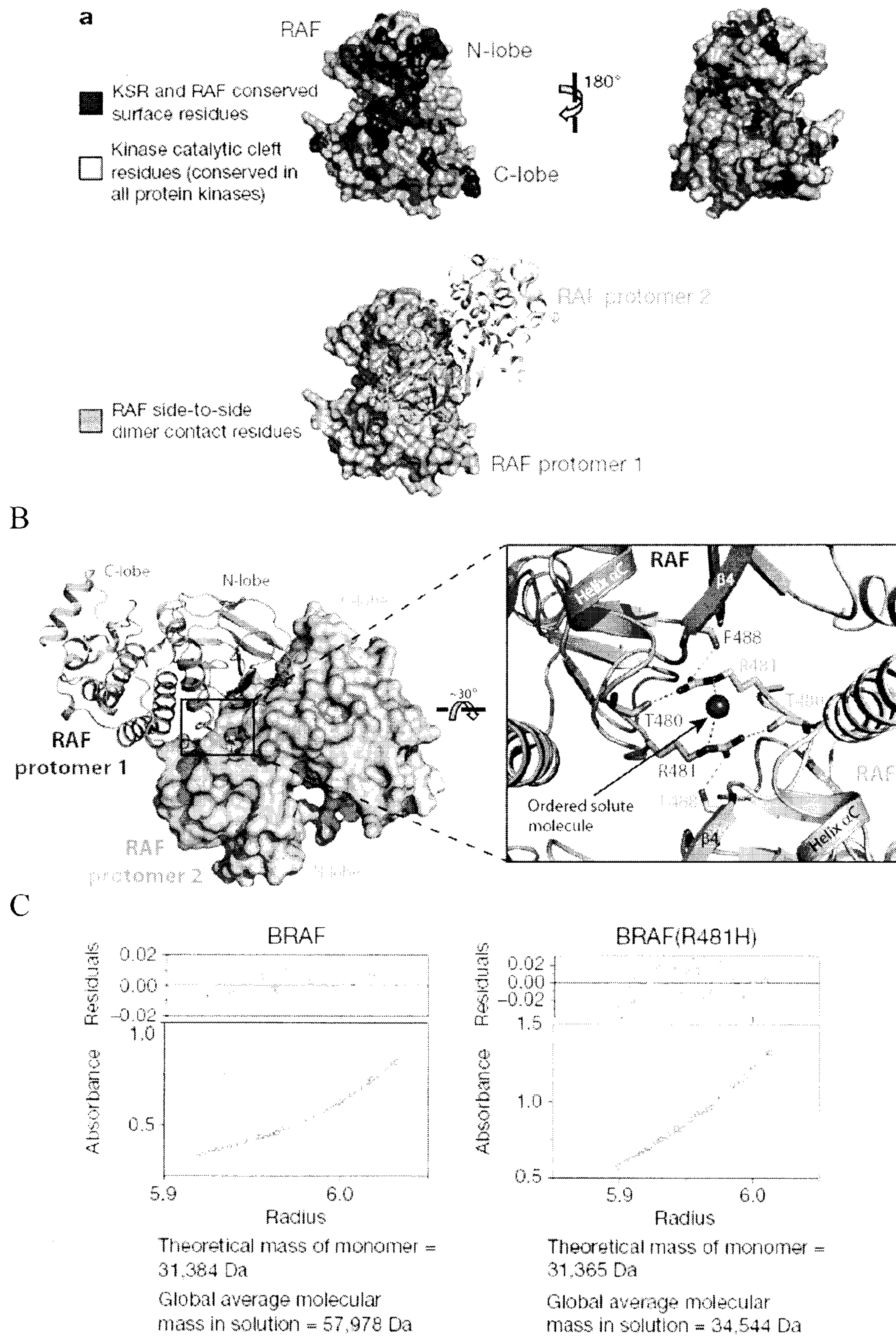


Figure 3

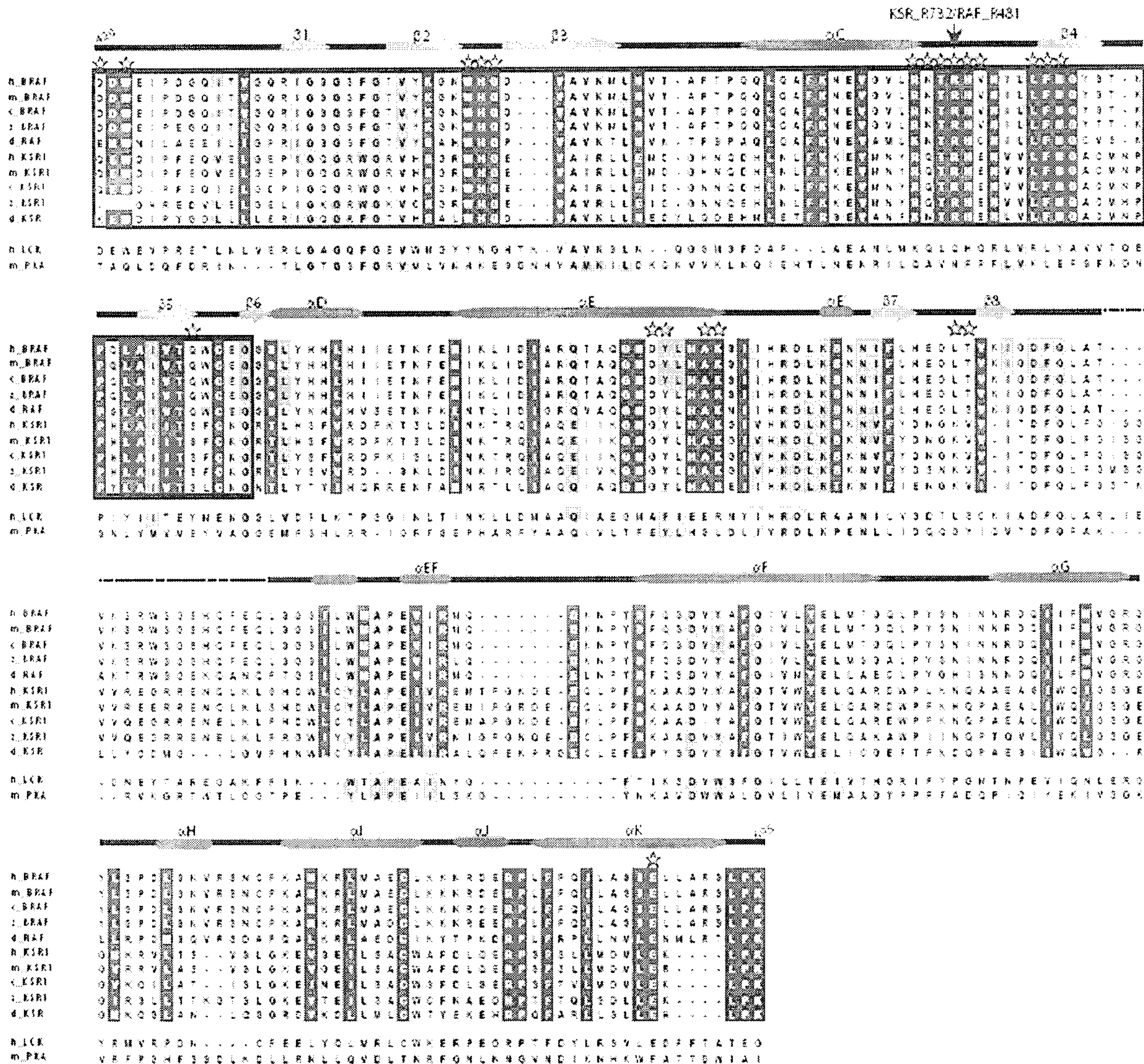
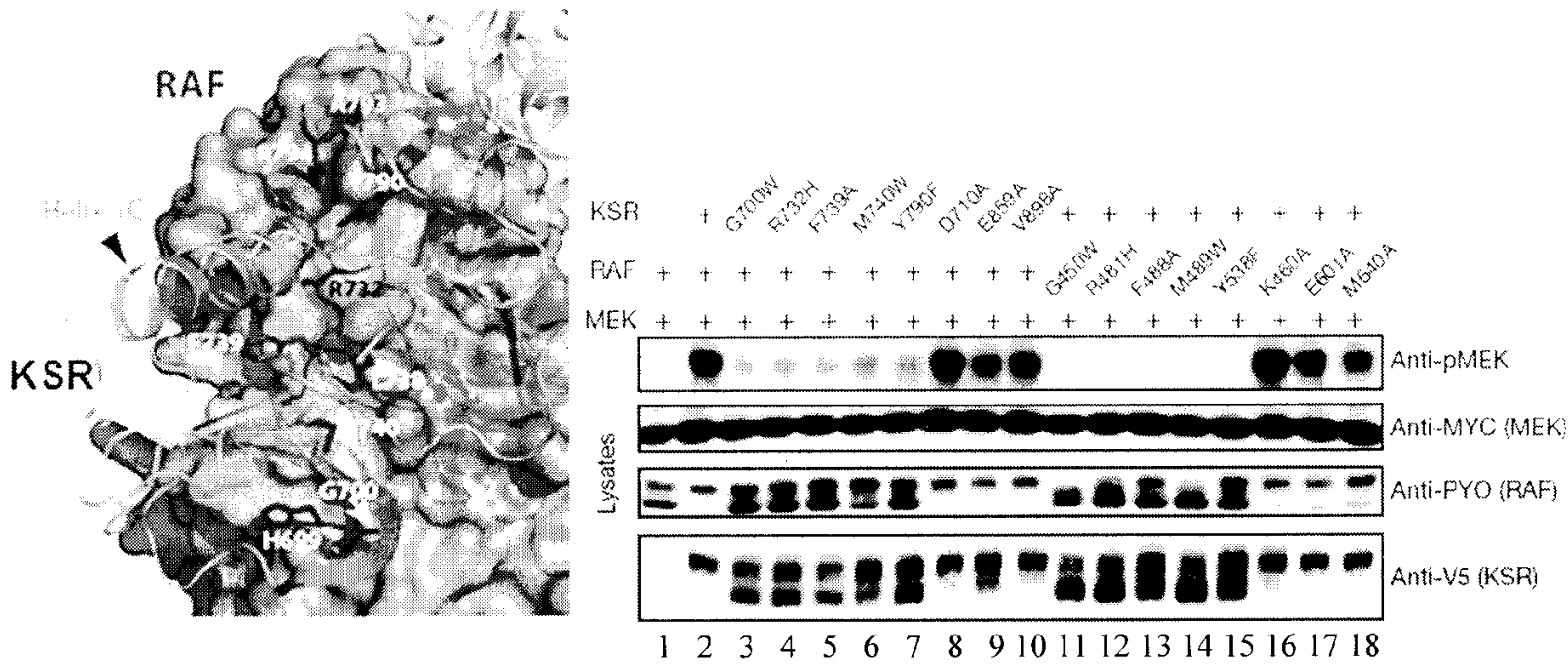
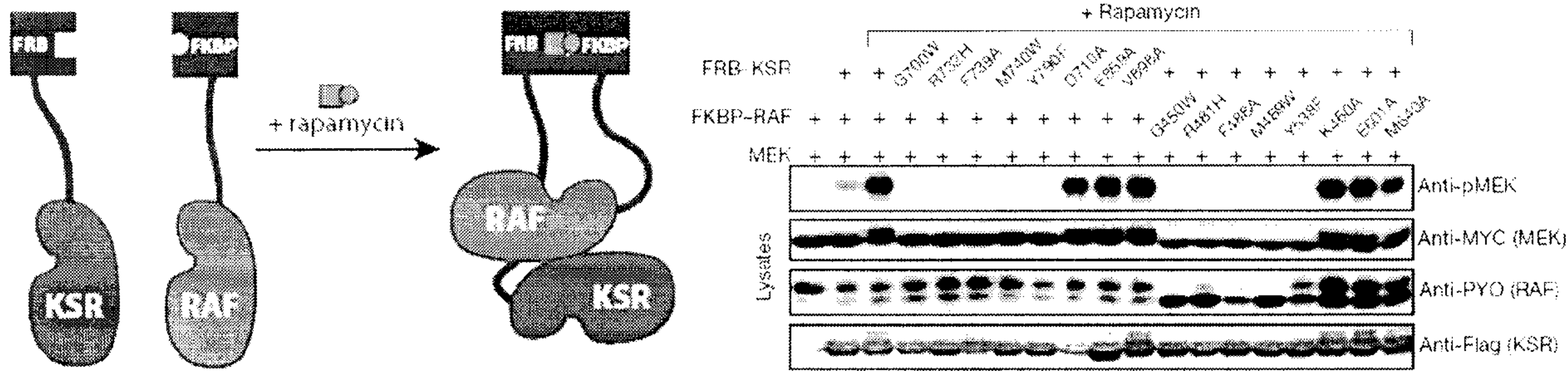


Figure 4

A



B



C

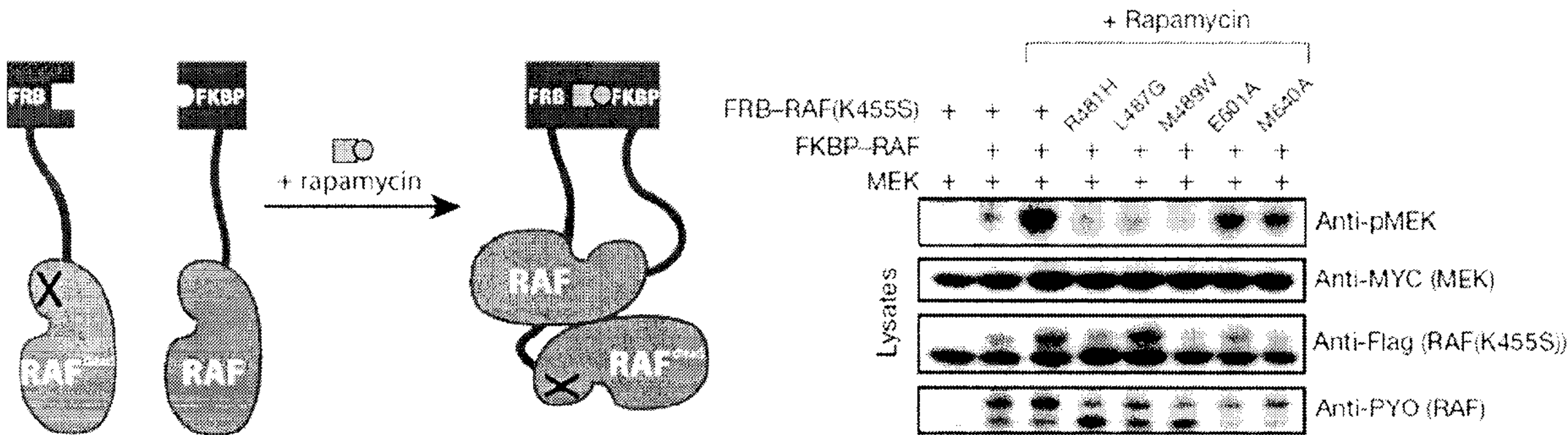


Figure 5

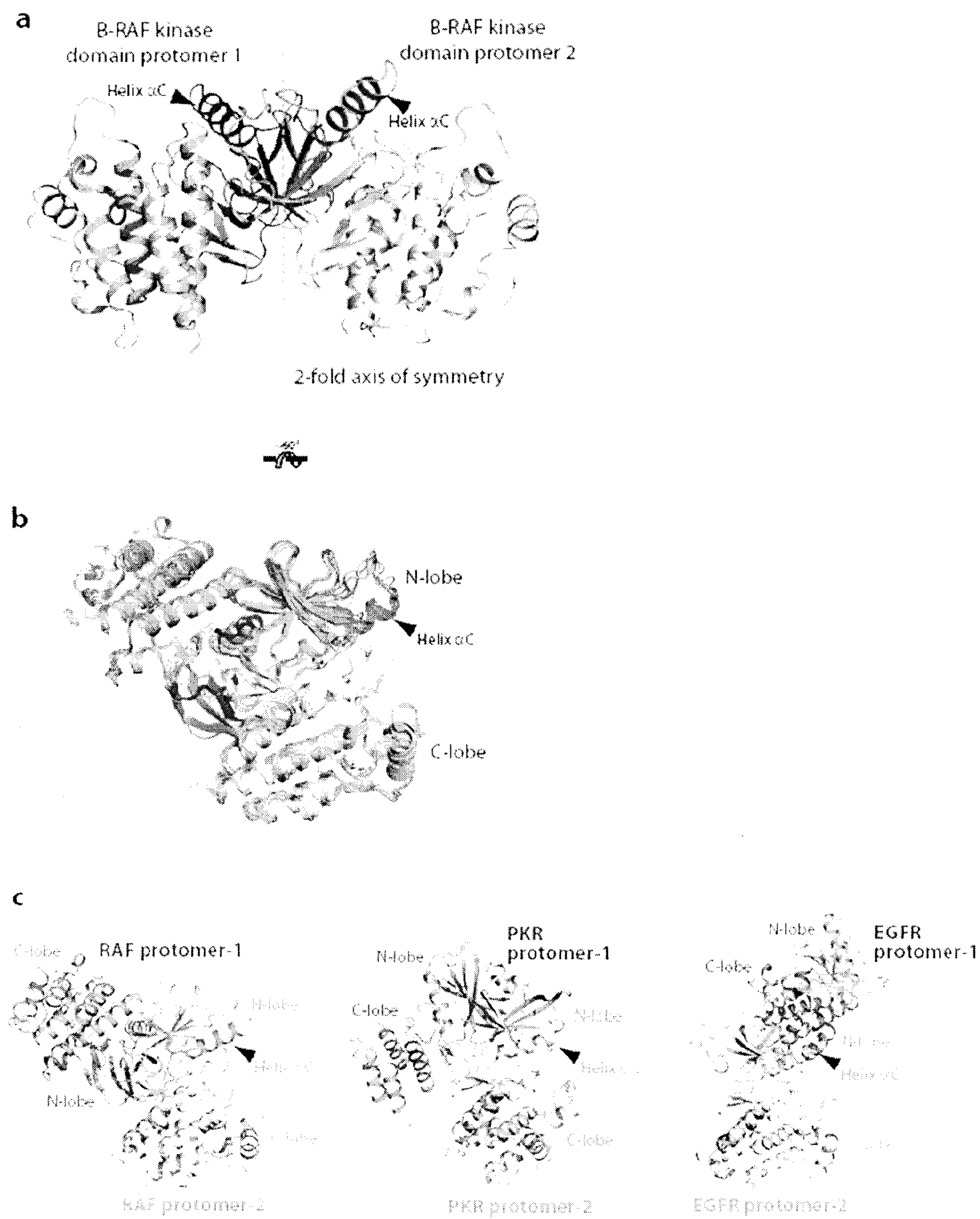


Figure 6

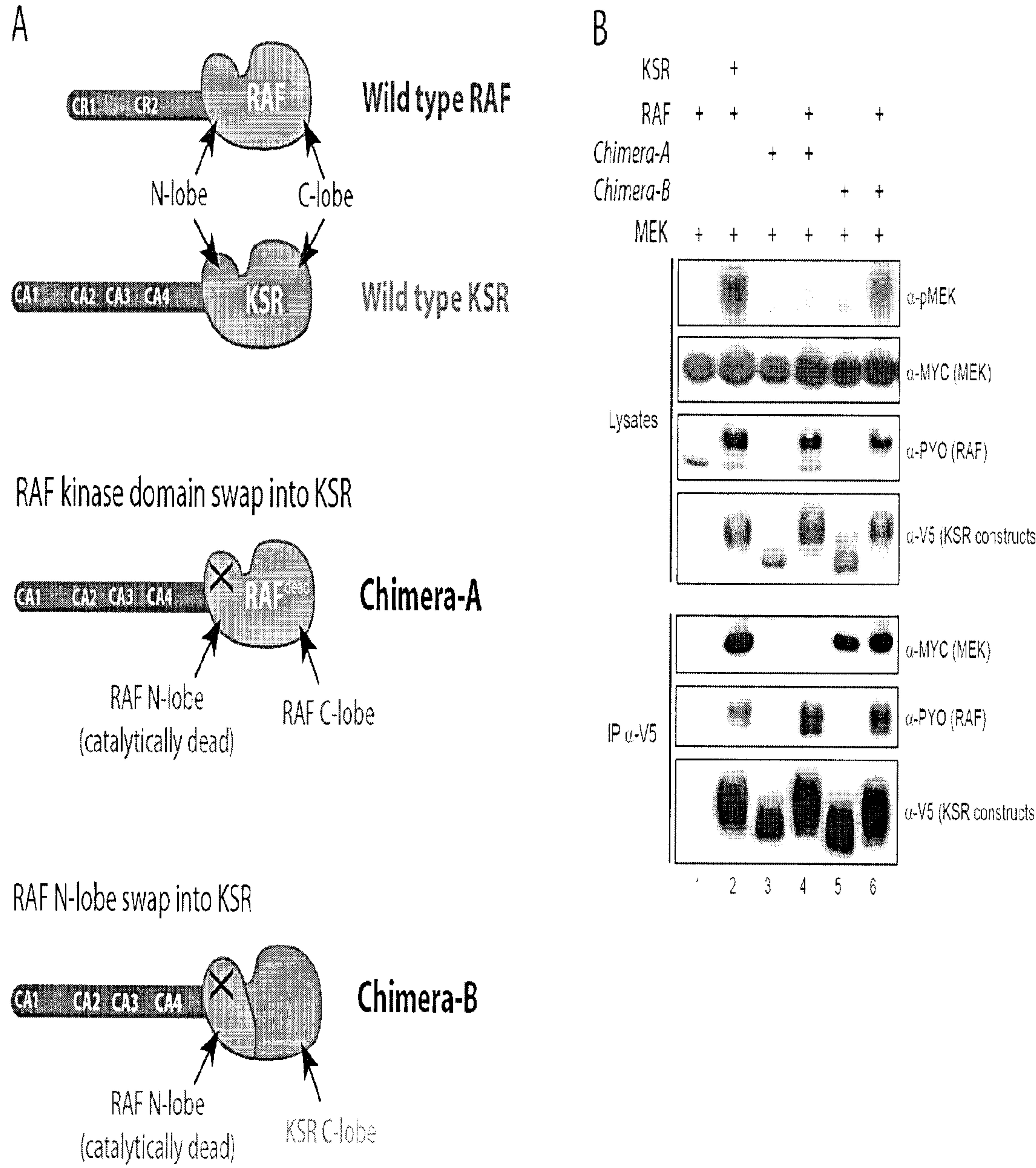


Figure 7

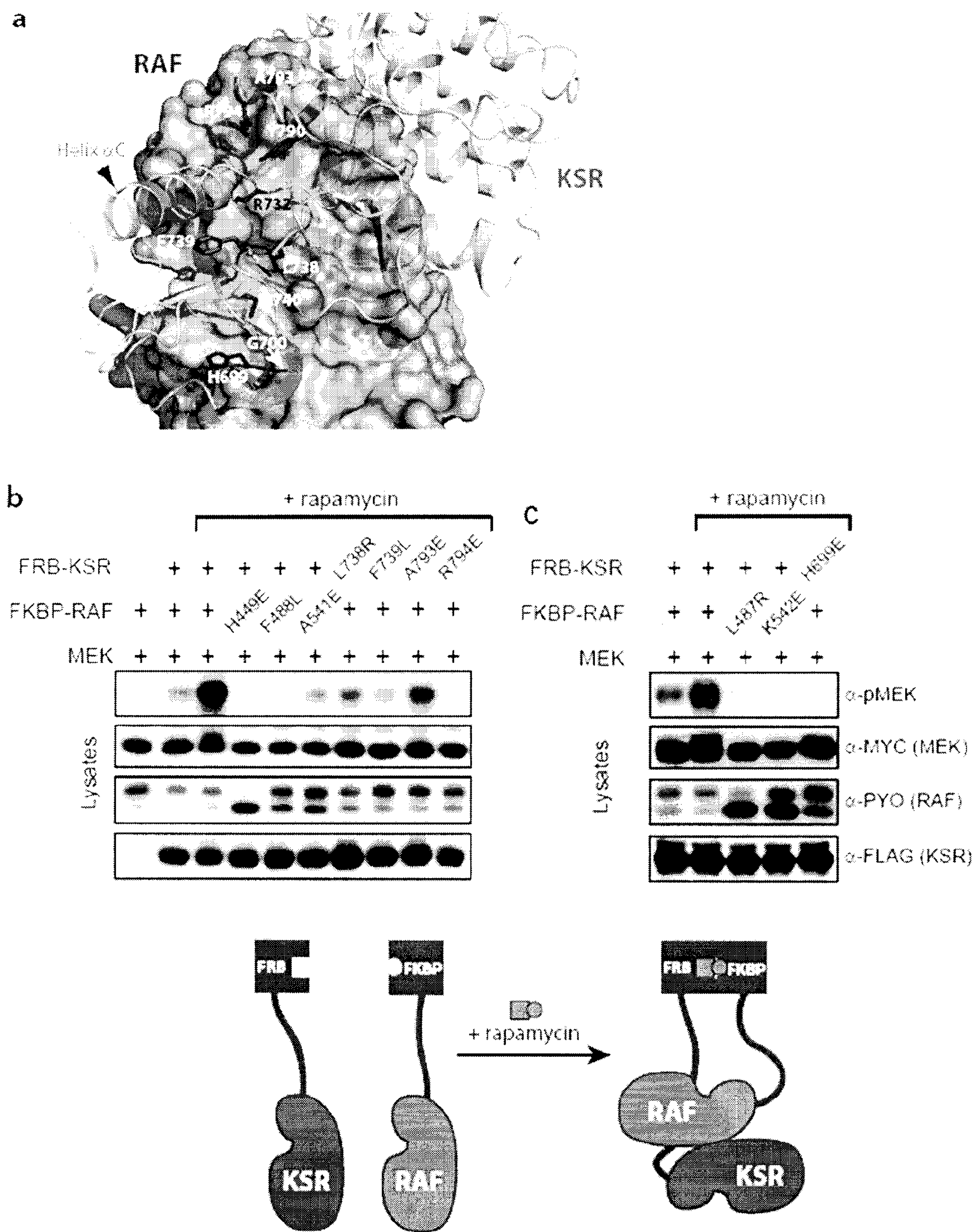


Figure 8

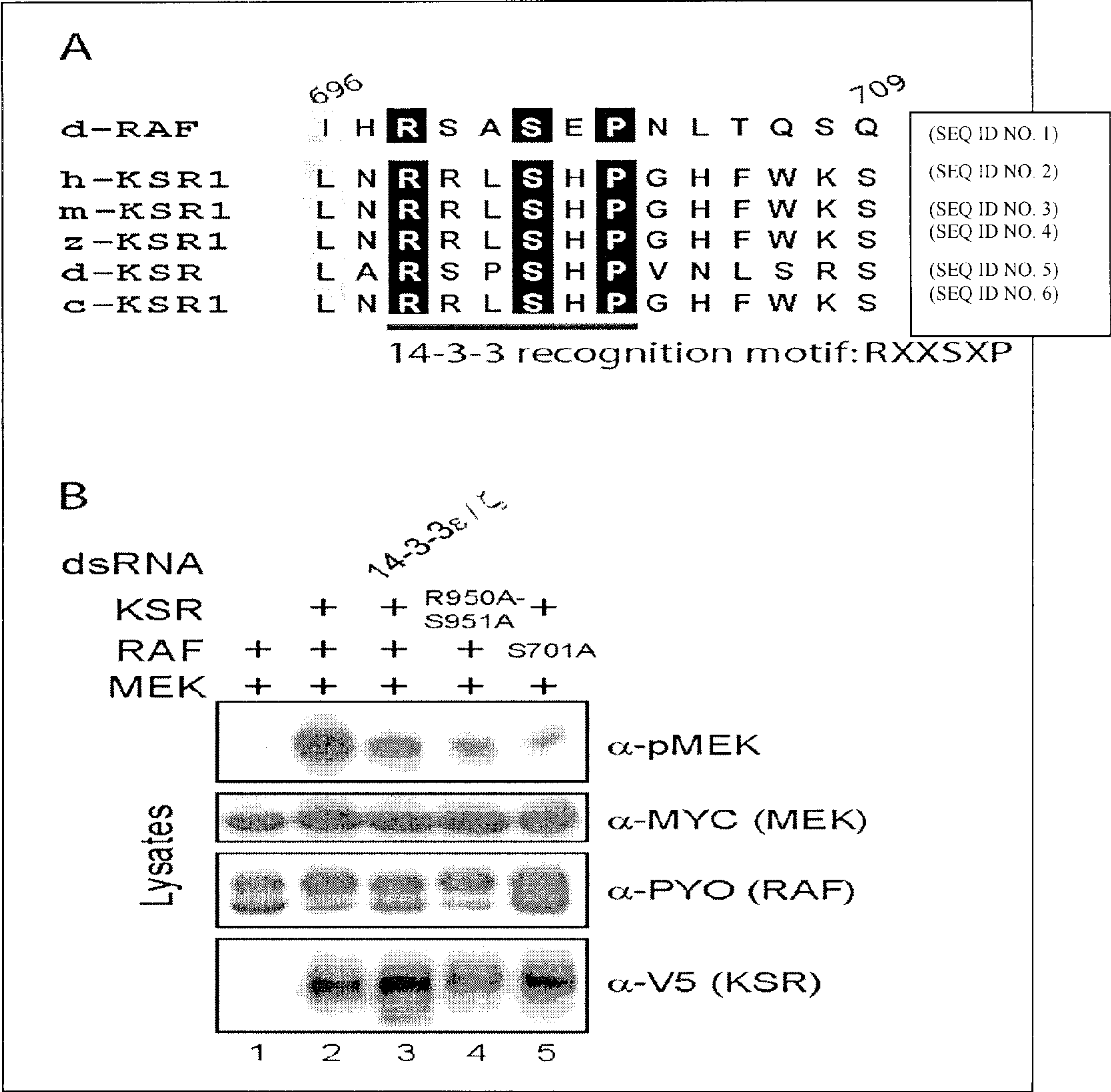


Figure 9

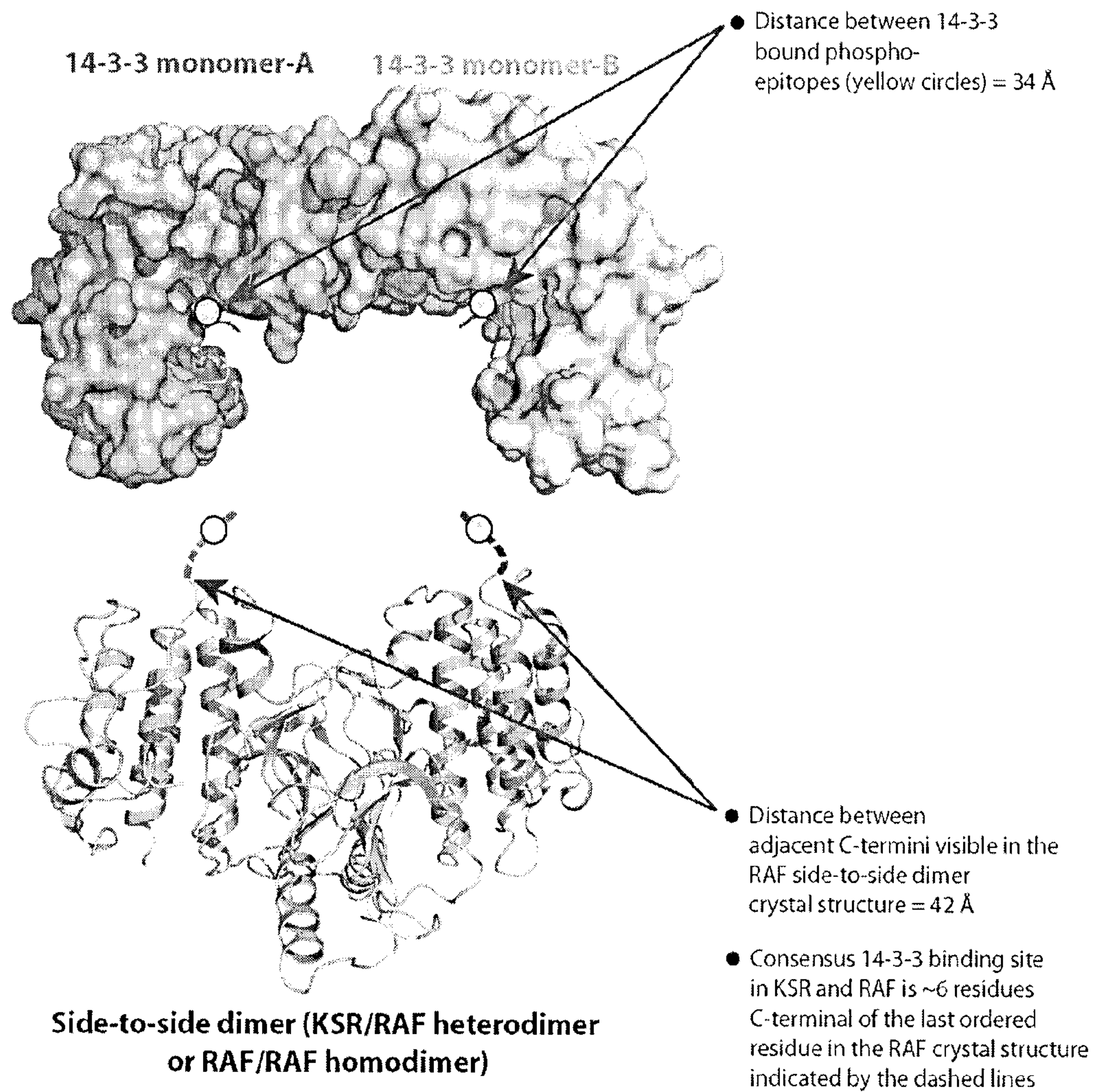


Figure 10

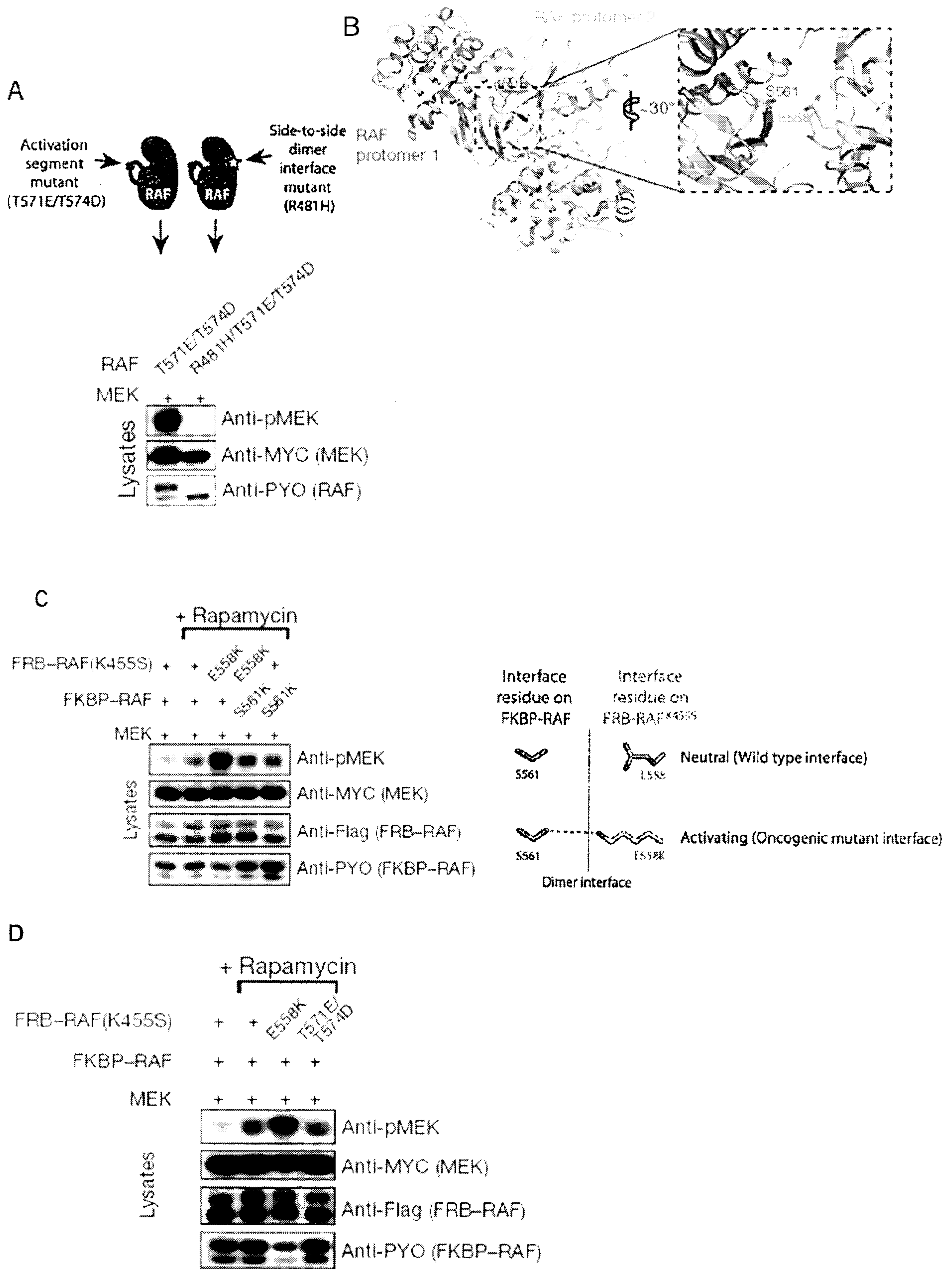


Figure 11

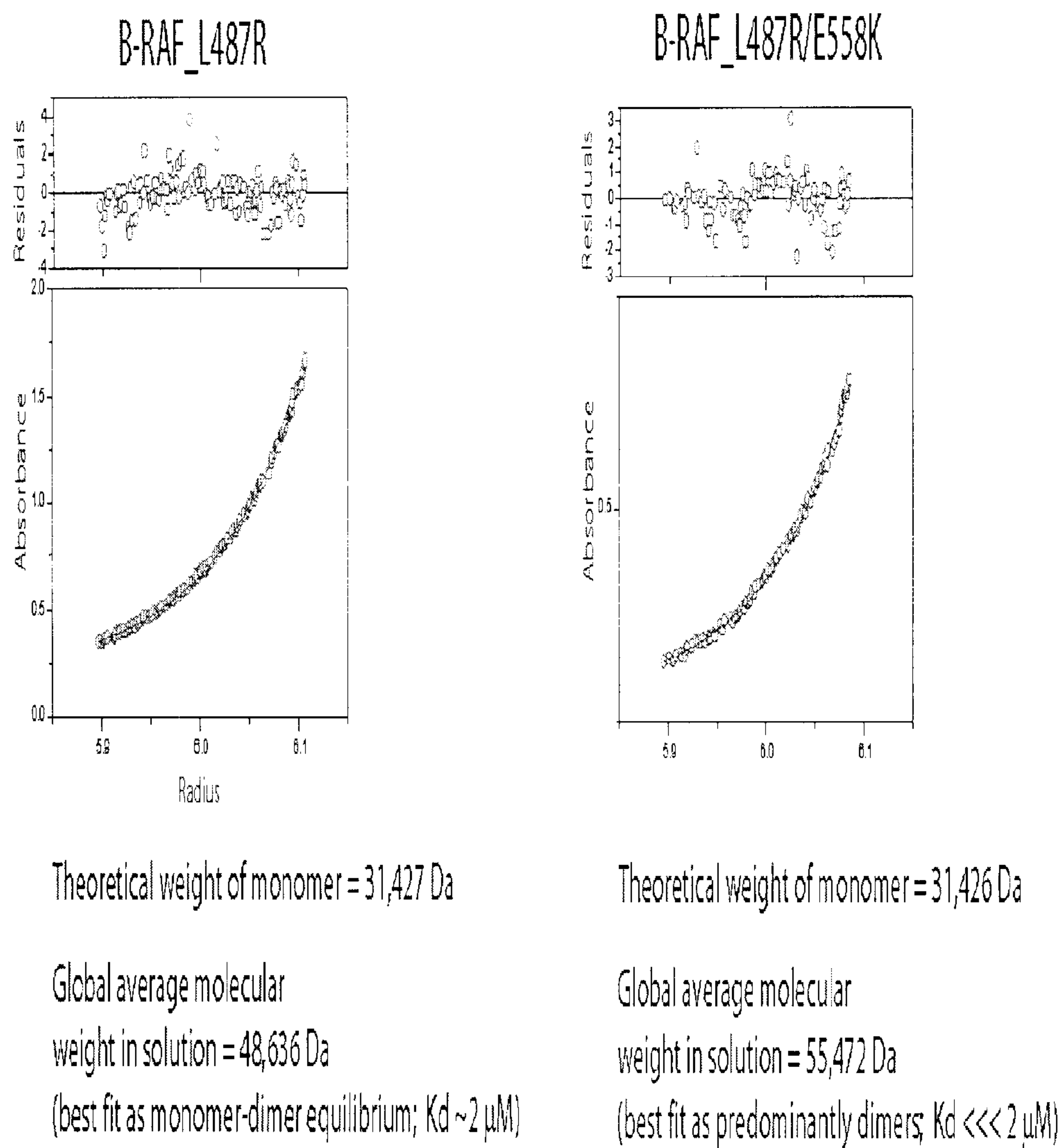


Figure 12

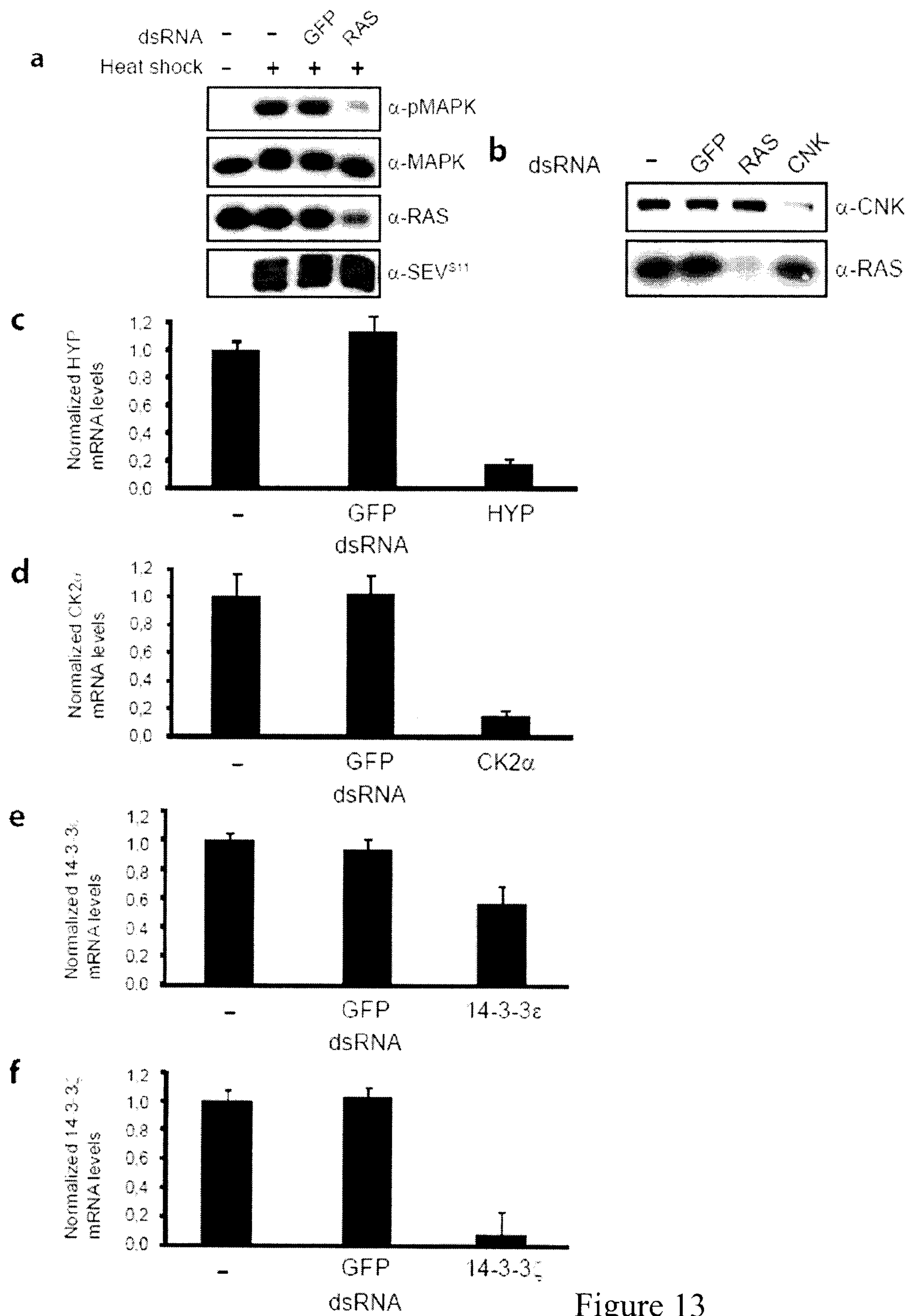


Figure 13

Homo sapiens v-raf murine sarcoma viral oncogene homolog B1 (BRAF), mRNA

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 GEELHVEVLENVPLTTHNFVRKTFFTLAFCDFCRKLLFQGFRCQTCGYKFHQRCSTEV
 PLMCVNYDQLDLLFVSKFFEHHPIPQEEASLAETALTSGSSPSAPASDSIGPQILTSP
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 GSTTGLSATPPASLPGSLTNVKALQKSPGPQREKSSSSSEDRNRMKTLGRRDSSDDW
 EIPDGQITVGQRIGSGSFGTVYKKGKWHGDVAVKMLNVTAPTQQQLQAFKNEVGVL~~RKT~~
~~RHVNILFM~~GYSTKPQLAIVTQWCEGSSLYHHLHIIETKFEMIKLIDIARQTAQGM~~DY~~
 LHAKSIIHRDLKSNNIFLHED~~LT~~VKIGDFGLATVKSRWSGSHQFEQLSGSILWMAPEV
 IRMQDKNPYSFQSDVYAFGIVLYELMTGQLPYSNINNRDQIIIFMVGRGYLSPDLSKVR
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 EDFSLYACASPKTPIQAGGYGAFPVH (SEQ ID NO. 7)

ORIGIN

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121	cggggacatg	gagcccgagg	ccggcgcccg	cgccggcgcc	gcggcctctt	cggctgcgga
181	ccctgccatt	ccggaggagg	tgtggaatat	caaacaaatg	attaagttga	cacaggaaca
241	tatagaggcc	ctattggaca	aatttggttg	ggagcataat	ccaccatcaa	tatatctgga
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361	ggaatctctg	gggaacggaa	ctgatttttc	tgtttctagc	tctgcatcaa	tggataccgt
421	tacatcttct	tcctcttcta	gcctttcagt	gctaccttca	tctctttcag	tttttcaaaa
481	tcccacagat	gtggcacgga	gcaaccccaa	gtcaccacaa	aaacctatcg	ttagagtctt
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601	tctaaagaaa	gcactgatga	tgagaggtct	aatcccagag	tgctgtgctg	tttacagaat
661	tcaggatgga	gagaagaaac	caattgggtg	ggacactgat	atttcctggc	ttactggaga
721	agaattgcat	gtggaagtgt	tggagaatgt	tccacttaca	acacacaact	ttgtacgaaa
781	aacgtttttc	accttagcat	tttgtgactt	ttgtcgaaag	ctgcttttcc	agggtttccg

Figure 14A

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901 tgttaattat gaccaacttg atttgctgtt tgtctccaag ttctttgaac accacccaat
961 accacaggaa gaggcgtcct tagcagagac tgccctaaca tctggatcat ccccttccgc
1021 acccgctcg gactctattg ggcccaaagt tctcaccagt ccgtctcctt caaatccat
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1201 tgacttgatt agagaccaag gatttcgtgg tgatggagga tcaaccacag gtttgtctgc
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1561 tgaagtagga gtactcagga aaacacgaca tgtgaatata ctactcttca tgggctatcc
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1741 tgcacagggc atggattact tacacgccaa gtcaatcata cacagagacc tcaagagtaa
1801 taatatattt ctcatgaag acctcacagt aaaaataggt gattttggtc tagctacagt
1861 gaaatctcga tggagtgggt cccatcagtt tgaacagttg tctggatcca ttttgtggat
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2941 ttataaaaa (SEQ ID NO. 8)

```

Figure 14A cont.

Mus musculus Braf transforming gene (Braf), mRNA

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EFAAFK" (SEQ ID NO. 9)

ORIGIN

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Figure 14B

```

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2341 gccatgaaga gattaatggc agagtgcctc aaaaagaaaa gagacgagag accactctt
2401 ccccaaattc tcgcctccat tgagctgctg gcccgctcat tgccaaaaat tcaccgcagt
2461 gcatcagaac cttccttgaa tcgggctggg ttccaaacag aagatttttag tctgtatgct
2521 tgtgcttctc cgaaaacacc catccaagca gggggatatg gagaatttgc agccttcaag
2581 tagccagtc ccatgtggcag catctactct ttatttctta agtcttgtgt tcatacagtt
2641 tgtaaacatc aaaacacagt tctgttcttc aaaaaatttt ttaaagatac aaaattttca
2701 atgcataagt tcatgtggaa cagaatggaa ttctctatc aacaaaagag ggaagaatgt
2761 tttaggaacc agaattctct gctgcccgtg tttcttcttc aacataacta tcacgtgcat
2821 acaagtctgc ccattcccaa gaagaaagag gagagaccct gaattctgcc cttttggtgg
2881 tcaggcatga tggaaagaat ttgctgctgc agcttgggaa aattgctatg gaaagtctgc
2941 cagtcgactt tgcccttcta accaccagat cagcctgtgg ctggtcatct gatggggcga
3001 tttccatcac caagcatcgt tcttgccctat tctgggatta tgttgtggag cactttccct
3061 gtccagcacc gttcatttct gagggatgga gtaaatgcag cattcccttg tgtagcgcct
3121 gttcagtcct cagcagctgc tgtcacagcg aagcttttta cagttaagtg gtgggggaga
3181 gttgaggaga gcctgcctcg gggcagagaa aaggggggtgc tgcactctct tcctcacctc
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3301 agtgctggca cacattgcct tcttttctca ttgggtccag caattgagga gaggggtggg
3361 ggattgtttc ctctcaatg tagcaaattc tcaggaaaat acagtccata tcttctctc
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3601 agaagaaaat aattttcagt ttaattgga ataaatgagt acttcccaca agactatata
3661 cctgaaaat tatatttttg ttaattgtaa acaactttta aagaataatt attatccttt
3721 tctctacct aaaaattatg ggaatcttag cataatgaca attatttata ctttttaaat
3781 aaatggta ctgtggatcc aactaacat ctttgctaac aatcccattg tttcttccaa
3841 cttaactcct aactacatc ctacatcctc tttctagtct tttatctata atatgcaacc
3901 taaaataaac gtgggtggcg ctccattcat tctccctctt cctgttttcc ccaagcctgg
3961 tcttcaaaag gttgggta atcggtccctga gctccctagc tggcaatgca actattaggg

```

Figure 14B cont.

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4021 acattggagt tgcaggagag caggaagcct gtccccagct gttcttctag aaccctaaat
4081 cttatctttg cacagatcaa agtatcacc tcgtcacagt tctccttagc ctttacttac
4141 aggtaatatata aataaaaatc accatagtag taaagaaaac aactggatgg attgatgacc
4201 agtacctctc agagccagga atcttgaatc tccaggattt atacgtgcaa atttaaggag
4261 atgtacttag caacttcaag ccaagaactt ccaaaatact agcgaatcta aaataaaatg
4321 gaattttgag ttatttttaa agttcaaatt ataattgata ccactatgta ttttaagccta
4381 ctcacagcaa gttagatgga ttttgctaaa ctcattgcc a gactgtggtg gtggtggtgg
4441 tagtgtgcac ctttaatcca agcaactcag caatcagaat gaggtaaatc tctgtgaata
4501 caaggcctgc ctagtctgca gcgctagtgc caggatagcc agggctacac acacaaaaac
4561 cctctctcaa aaaaaacaaa attaatagtg tgataataaa aaataactaa agtatcatca
4621 aaggaaggcc tactggaagt tttatatatt cccagtaa at tgaaaaatat tctgaagtta
4681 ttaaccagtt agcaacaatg tgtttttaag tcttacataa acagagcaaa gtcttcaa at
4741 gtttcagagc tgagaagata attgtgcttg atatgaaaaa tagcctctcc atatgatgtg
4801 ccacattgaa aggcgtcatt acccttttaa atacttctta atgtggcttt gtcccttta
4861 cccaggatta gctagaaaga gctaggtagg cttcggccac agttgcacat ttcgggctg
4921 ctgaagaatg ggagctttga aggcctggcct tgggtggagga gccctcagt gctggagggt
4981 ggggcgtgta cgcagcatgg aagtggctca gacagagtgc aaaggacag acttcttct
5041 catttttagta taggggtgat tctcacttga aatgagaaag tagagttgat attaaacgaa
5101 gctgtgcca gaaaccaggc tcagggtatt gtgagatttt ctttttaaat agagaatata
5161 aaagatagaa ataaatat tt aaaccttcc tcttattttc tatcaaatag atttttttta
5221 tcatttgcaa acaacataaa aaaaggtttc ttttggtggg ttttctttcc ttcttttttt
5281 tttttttttt tttttaagac tgcagataat cttgttgagc tctcggaaa atacaaggaa
5341 gtccgtgttt gtgcagagcg ctttatgagt aactgtatag acagtgtggc tgcttctctc
5401 atcccagagg gctgcagctg tcggcccatg aagtggctgc agtgccctgt gagatctgct
5461 ttgttttggt tggagtgaag tctttgaaag gtttgagtgc aactatatag gactgttttt
5521 aaataagtag tattcctcat gaactttctc attgttaagc tacaggacc aaactctacc
5581 actaagatat tattaacctc aaaatgtagt ttatagaagg aatttgcaaa tagaatatcc
5641 agttcgtact tatatgcac tcaacaaaag attctctgtg acttggtgga tttgggtcct
5701 gaacagccca tttctgtatt tgagggttagg agggcataat gaggcacct aaaagacaat
5761 ctgatataaa ctgtatgcta gatgtatgct ggtaggggag aaagcattct gtaaagacat
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6361 ctttgctagc catgatataa ttctactttt taggagtagg tttggcaaaa ctgtatgcct
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6481 agctaagagg gcggaaggat accctttttt cctacgattc gcttctttgt ccacgttggc
6541 attgttagta ctagtttatc agcaccttga ccagcagatg tcaaccaata agctattttt
6601 aaaaccatag ccagagatgg agaggctact gtgagtagaa acagcaggac gcttacagga
6661 gtgaaatggt gtagggaggc tctagaaaaa tatcttgaca atttgcaaaa tgatcttact
6721 gtgccttcat gatgcaataa aaaagctaac attttagcag aaatcagtga tttacgaaga
6781 gagtggccag tctggtttaa ctcagctggg ataatatatt tagagtgcaa ttttagactgc
6841 gaagataaat gcactaaaga gtttatagcc aattcacatt tgaaaaataa gaaaatggta

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Figure 14B cont.

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6901 aatttttcagt gaaatatttt tttaaagcac ataatecccta gtgtagccag aaatattttac
6961 cacatagagc agctaggctg agatacagtc cagtgcacatt tctagagaaa ccttttctac
7021 tcccacgggc tctcaaaagc atggaaattt tatacaaaat gtttgacatt ttaagatact
7081 gctgtagttt agttttgaaa tagtatgtgc tgagcagcaa tcatgtacta actcagagag
7141 agaaaacaac aacaaattgt gcatctgatt tgttttcaga gaaatgctgc caacttagat
7201 actgagttct cagagcttca agtgtaaact tgcctcccaa gtctgtttg caaatgaagt
7261 tggctagtgc tactgactgc tccagcacat gatggaaggc agggggctgt ctctgaagtg
7321 tcttctataa agggacaata gaatagttag agacctggtc agtgtgtgtc agctggacac
7381 tccatgctat gggacttgca tcttctgtcc tcaccatccc caagacattg tgctttcctc
7441 agttgtcctc tagctgtttc actcagacac caagatgaat tactgatgcc agaagggggc
7501 aaaatggcca gtgtgttttg ggggttgat cagttgactg gacaataact ttaatagtgt
7561 cagatcattt atttttactt ccattttgac agacatttaa atggaaattt agtccctaact
7621 tttgtcattt gaaaggaaaa attaacagtt cctataagat acttttgagg tggaatctga
7681 catcctaatt ttttttcttt tcagtgggtt tgcagcgagg gtcttgtagt cactaggcaa
7741 gggttctacc actaagccac atttcccagg aaataaaatg ttaacagtta aaacatacac
7801 acaatacac aaacacctta ttaccacttt agtaaagtga gagatgtgcg tcctttgtct
7861 cagtctccac gatttcagct gcccttgta tgaataactc agtctcgcta aactgtttac
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7981 ccagtgtgtt tgttttggtt ttgggttttt gtggtacatt tttgttaaag aattctgtag
8041 attgaagtgc tctttgaaaa cagaactgag atatatttat tcttgtttag atcaaaaaac
8101 attttggtgca aatgatttgc ttttcctggc aggctgagta ccatatccag cgcccacaat
8161 tgcgggttcc catctacat gtccacaggg gagacagacg ggaagcacat gaggggtgtg
8221 tttacagagt ttaggaggtt atgtagtctt ctgttgctt tggaatcac tgtgtttta
8281 agactgttga acccggtgtg ttggctgggc tgtgagttac atgaagaaac tgcaacttag
8341 catatgcaga caaagctcac agactaggcg taaatggagg aaaatggacc aaaataaggc
8401 agggtgacac ataaaccttg ggcttcggag aaaactaagg gtggagatga actataatca
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8521 atcattcctt tctaatacat ttatgtatgt tccattgctg actaaaacca gctatgagaa
8581 catatgcctt tttattcatg ttaactacca gtttaagtgg ctaaccttaa tgtcttattt
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9121 accacccgca cctatggcaa cccatataca ttcctcttgt ctgtaactgc caaagggttg
9181 gtttatgtca cttcagttcc actcaagcat tgaaaagggt ctcattggag ctgggggtgtg
9241 cccagtgaag agatggggac tttttcatta tccacagacc tctctatacc tgctttgcaa
9301 aaattataat ggagtaacta tttttaaagc ttatttttca attcataaga aaaagacatt
9361 tattttcaat caaatggatg atgtctctta tcccttatcc ctcaatgttt gcttgaattt
9421 tgtttgttcc ctatacctac tccctaattc tttagtctct tctgtctcag gtcccttcat
9481 ttgtactttg gagtttttct catgtaaatt tgtataatgg aaaatattgt tcagtttgga
9541 tagaaagcat ggagaaataa ataaaaaag atagctgaaa atcaaattga agaaatttat
9601 ttctgtgtaa agttatttaa aaactctgta ttatatttaa agaaaaaagc ccaaccccc
9661 aaaaagtgtc atgtaattga tgtgaatatg cgaatactgc tataataaag attgactgca
9721 tggagaaa (SEQ ID NO. 10)

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Figure 14B cont.

Homo sapiens kinase suppressor of ras 1 (KSR1), mRNA

MDRAALRAAAMGEKKEGGGGDAAEGGAGAAASRALQQCGQLQKLIDISIGSLRGLRTKC
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 VQEIPRDLTLDALLEMNEAKVKETLRRCGASGDECGRLQYALTCLRKVTGLGGEHKEDS
 SWSSLDARRESGSGPSTDTLSAASLPWPPGSSQLGRAGNSAQGPRSISVSALPASDSP
 TPSFSEGLSDTCIPLHASGRLTPRALHSFITPPTTPQLRRHTKLKPPRTPPPPSRKVF
 QLLPSFPTLTRSKSHESQLGNRIDDVSSMRFDLSHGSPQMVRDIGLSVTHRFSTKSW
 LSQVCHVCQKSMIFGVKCKHCRLKCHNKCTKEAPACRISFLPLTRLRRTESVPSDINN
 PVDRAAEPHFGLPKALTKEHPPAMNHLDSSSNPSSTTSSTPSSPAPFPTSSNPSSA
 TTPPNPSPGQDSRFNFPAAYFIHHRQQFIFPVPSAGHCWKCLLIAESLKENAFNISA
 FAHAAPLPEAADGTRLDDQPKADVLEAHEAEAEPEAGKSEAEDDEDEVDDLPSRRP
 WRGPISRKASQTSVYLQEW~~DI~~PF~~EQ~~VELGEPIGQGRWGRVHRGR~~WH~~GEVAIRLLEMDG
 HNQDHLKLFKKEVMNY~~RQ~~~~TR~~~~HE~~NV~~LF~~MGACMNPPHLAIIT~~S~~FCKGRTLHSFVRDPKT
 SLDINKTRQIAQEIIKGM~~GY~~L~~AK~~GIVHKDLKSKNVFYDNG~~KV~~VITDFGLFGISGVVR
 EGRRENQLKLSHDWLCYLAPEIVREMTPGKDEDQLPFSKAADVYAFGTVWYELQARDW
 PLKNQAAEASIWQIGSGEGMKRVLTSVSLGKEVSEILSACWAFDLQERPSFSLMDML

EKLPKLNRRLSHPGHFWKSAEL (SEQ ID NO. 11)

ORIGIN

1	ctggaccct	gccaggaag	gggtcctcag	acttgagggt	gccagctcag	atgtggggct
61	gctgatacta	ggtgactgga	ctgatgttct	gttctagatg	aaactccttg	aggggacat
121	ttgaaaagc	ttgatgtgct	gccccaaagc	cccttcagag	ctgacttctc	cacccccagc
181	tgccgtgagc	cttggtgct	gacagctcat	agctgagtc	ctcccgtgaa	gtcaccttct
241	gctgaagggt	acatcctctc	ccaaggcgaa	gctgggtccgt	tacatttgta	agcagaggca
301	gtgcaagctg	agcgtggctc	ccggtgagag	gaccccagag	ctcaacagct	accccgcctt
361	cagcgactgg	ctgtacactt	tcaacgtgag	gccggagggtg	gtgcaggaga	tcccccgaga
421	cctcacgctg	gatgccctgc	tggagatgaa	tgaggccaag	gtgaaggaga	cgctgcggcg
481	ctgtggggcc	agcgggggatg	agtgtggccg	tctgcagtat	gccctcacct	gcctgcggaa
541	ggtgacaggc	ctgggagggg	agcacaagga	ggactccagt	tggagttcat	tggatgcgcg
601	gcgggaaagt	ggctcagggc	cttccacgga	cacctctca	gcagccagcc	tgccctggcc
661	cccagggagc	tcccagctgg	gcagagcagg	caacagcgcc	cagggccac	gctccatctc
721	cgtgtcagct	ctgcccgcct	cagactcccc	cacccccagc	ttcagtgagg	gcctctcaga
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841	caccccgccc	accacacccc	agctgcgacg	gcacaccaag	ctgaagccac	cacggacgcc
901	ccccccaccc	agccgcaagg	tcttcagct	gctgcccagc	ttccccacac	tcacccggag
961	caagtcccat	gagtctcagc	tggggaaccg	cattgatgac	gtctcctcga	tgaggtttga

Figure 14C

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1021 tctctcgcat ggatccccac agatggtacg gagggatata gggctgtcgg tgacgcacag
1081 gttctccacc aagtcctggc tgtcgcaggt ctgccacgtg tgccagaaga gcatgatatt
1141 tggagtgaag tgcaagcatt gcagggtgaa gtgtcacaac aaatgtacca aagaagcccc
1201 tgcctgtaga atataccttc tgccactaac tcggcttcgg aggacagaat ctgtcccctc
1261 ggacatcaac aacccggtgg acagagcagc cgaaccccat tttggaacce tccccaaagc
1321 actgacaaag aaggagcacc ctccggccat gaatcacctg gactccagca gcaacccttc
1381 ctccaccacc tctccacac cctcctcacc ggcgcccttc ccgacatcat ccaacccttc
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1501 agctgcctac ttcattcatc atagacagca gtttatcttt ccagtgccat ctgctggcca
1561 ttgctggaaa tgcctcctta ttgcagaaag tttaaaggaa aacgctttca acatttcagc
1621 ctttgcacac gcagccccgc tccctgaagc tgccgacggg acccggtctg atgaccagcc
1681 gaaagcagat gtgttggaag ctacgaagc ggaggctgag gagccagagg ctggcaagtc
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2161 gacgaggcaa atcgctcagg agatcatcaa gggcatggga tatcttcatg ccaagggcat
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2281 agacttcggg ctgtttggga tctcaggcgt ggtccgagag ggacggcgtg agaaccagct
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2581 gaaggaagtc agtgagatcc tgtcggcctg ctgggctttc gacctgcagg agagaccag
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Figure 14C cont.

```
3961 ggggcagctc tgccagggct gttggccaat cagtcatttt catttcttgt tggaggccag
4021 gtcctctgct gaactcattt cctagctagt gttaccctaa ttctgatgaa gatcaatggg
4081 gctataattc ttgtttttgt tcttctttgc agcattaaca gcagcaaagt tgtaccccg
4141 tttgaaagggt ttggcttggg cgtcctggag tccagtaatc caaagatgta gccagccata
4201 tggtttttcg ctgctgatct ctttcttttt aaaatgtgtt tctgaaacat cccaacaacc
4261 accacgacaa aaaaacactg cctgcccagc gctgcaaacc aggagcacac gtcctagatt
4321 cagactgttg gccataaacc ccaactcggga gatggagctg cacctgctat ttcttaaaat
4381 gacaccacca acaaccaaac ctgtcatgac agacagcaaa tgtttacacg tatatttctc
4441 ctgagtgaac ctgatgtttt acaataggta ataataaaaa cagtctgtgc aaaaaaaaaa
4501 aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aa (SEQ
ID NO. 12)
```

Figure 14C cont.

Mus musculus kinase suppressor of ras 1 (Ksr1), mRNA

MDRAALRAAAMGEKKEGGGGGAAADGGAGAAVSRALQQCGQLQK

LIDISIGSLRGLRTKCSVSNDLTQQEIRTLEAKLVKYICKQQQSKLSVTPSDRTAELN

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RFSTKSWLSQVCNVCQKSMIFGVKCKHCRLKCHNKCTKEAPACRITFLPLARLRRTES

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SNPSSATTPPNPSPGQRDSRFSFPDISACSQAAPLSSTADSTRLDDQPKTDVLGVHEA

EAEPEAGKSEAEDDEEDEVDLPSRRPWRGPISRKASQTSVYLQEWDTPFQVELG

EPIGQGRWGRVHRGRWHGEVAIRLLEMDGHNQDHLKLFKKEVMNRYRQTRHENNVLEMG

ACMNPPHLAIITSFCKGRTLHSFVRDPKTSLDINKTRQIAQEIIKGMGYLHAKGIVHK

DLKSKNVFYDNGKVITDFGLFGISGVVREERRENQLKLSHDWLCYLAPEIVREMIPG

RDEDQLPFASKAADVYAFGTVWYELQARDWPFKHQPAEALIWQIGSGEGVRRVLASVSL

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RFERFGLGTLESGNPKM (SEQ ID NO. 13)

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121	gagggcgggc	gcgggggcgc	cgcggcggac	gggggcgcag	gggcgcgcgt	cagccggggc
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481	atggacgagg	ccaaagccaa	ggagatgctg	cggcgctggg	gggccagcac	ggaggagtgc
541	agccgcctac	agcaagccct	tacctgcctt	cggaagggtga	ctggcctggg	aggggagcac
601	aaaatggact	caggttggag	ttcaacagat	gctcgagaca	gtagcttggg	gcctcccatg
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721	gtgtccgccc	tgctgcctc	agactctccg	gtccccggcc	tcagtgagg	cctctcggac
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961	aagtcccacg	agtcccagct	gggaaaccga	atcgacgacg	tcaccccgat	gaagtttgaa
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1081	ttctccacaa	agtcattggt	gtcacaggtg	tgcaacgtgt	gccagaagag	catgattttt
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Figure 14 D

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1321 ctgacaaaga aggagcaccc tccagccatg aacctggact ccagcagcaa cccatcctcc
1381 accacgtcct ccacaccctc atcgccggca cctttcctga cctcatctaa tccctccagt
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3781 gagcgcgaga cctctgtgtt cattcctatg agctggaggg gctggactgg gtggggtcag
3841 atgtgcttgg caggaaactgt cagctgctga gcagggtggg ccctgagcgg aggataagca
3901 gcatcagact ccacaaccag aggaagaaag aaatggggat ggagcggaga cccacgggct
3961 gagtcccgt gtggagtggc cttgcagctc cctctcagtt aaaactccca gtaaagccac
4021 agttctccga gcacccaagt ctgctccagc cgtctcttaa aacaggccac tctctgagaa
4081 ggaattc (SEQ ID NO. 14)

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Figure 14D cont.

Drosophila melanogaster pole hole (phl), transcript variant A, mRNA

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 THIKHQIIRKTFSLVFCEGCRLLFTGFYCSQCNFRFHQRCANRVPMLCQPFPMDSY
 YQLLLAENPDNGVGFPGRGTAVRFNMSSRSRRCSSSGSSSSSKPPSSSSGNHRQGR
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 FGLATAKTRWSGEKQANQPTGSILWMAPEVIRMQELNPYSFQSDVYAFGIVMYELLAE
 CLPYGHISNKDQILEFMVGRGLLRPDMSQVRSDAPQALKRLAEDCIKYTPKDRPLFRPL
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 NO. 15)

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Figure 14E

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(SEQ ID NO. 16)

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Figure 14E cont.

Drosophila melanogaster kinase suppressor of ras (ksr), mRNA

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 HRQHGVKGKNSALANSTNFKSGRQSPSATEELNSTQGSQTLTLTPSPPNSPFTPSSG
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 HRWMKCTDCKYICHKSCAPHVPPSCGLPREYVDEFRIKEQGGYASLPHVHGAAKGSP
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 GACMNPPYLAIVTSLCKGNTLYTYIHQRREKFAMNRTLLIAQQIAQGMGYLHAREIIH
 KDLRTKNIFIENGKVIITDFGLFSSTKLLYCDMGLGVPHNWLCYLAPELIRALQPEKP
 RGELEFTPYSDVYSFGTVWYELICGEFTFKDQPAESI IWQVGRGMKQSLANLQSGRD
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Figure 14F

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3361 gagcactcca gttgcagttg ttagcaaacg aaacaaaggc aaatcaaata ttaactcgaa
3421 aaagacaaaa cgcttaaatg tttaagagca gaggcaaaca gagaaggcat agacatacat
3481 atacaaacaa acaaacaagc actgtggcaa acataaatgt aaacgttaat caggtgagca
3541 atttctaaat tgttaattat gtgtaagaga actatatata tatatatata tatatatata
3601 tatatatata tatatatata tgtatatata gcagcaatgt attgtatatg acggactagt
3661 gttaaattaa atatatattg tgaattatgt atggcgaagt gtatatagta aatggacttt
3721 aaatgcgaaa tcgaaac (SEQ ID NO. 18)

```

Figure 14F cont.

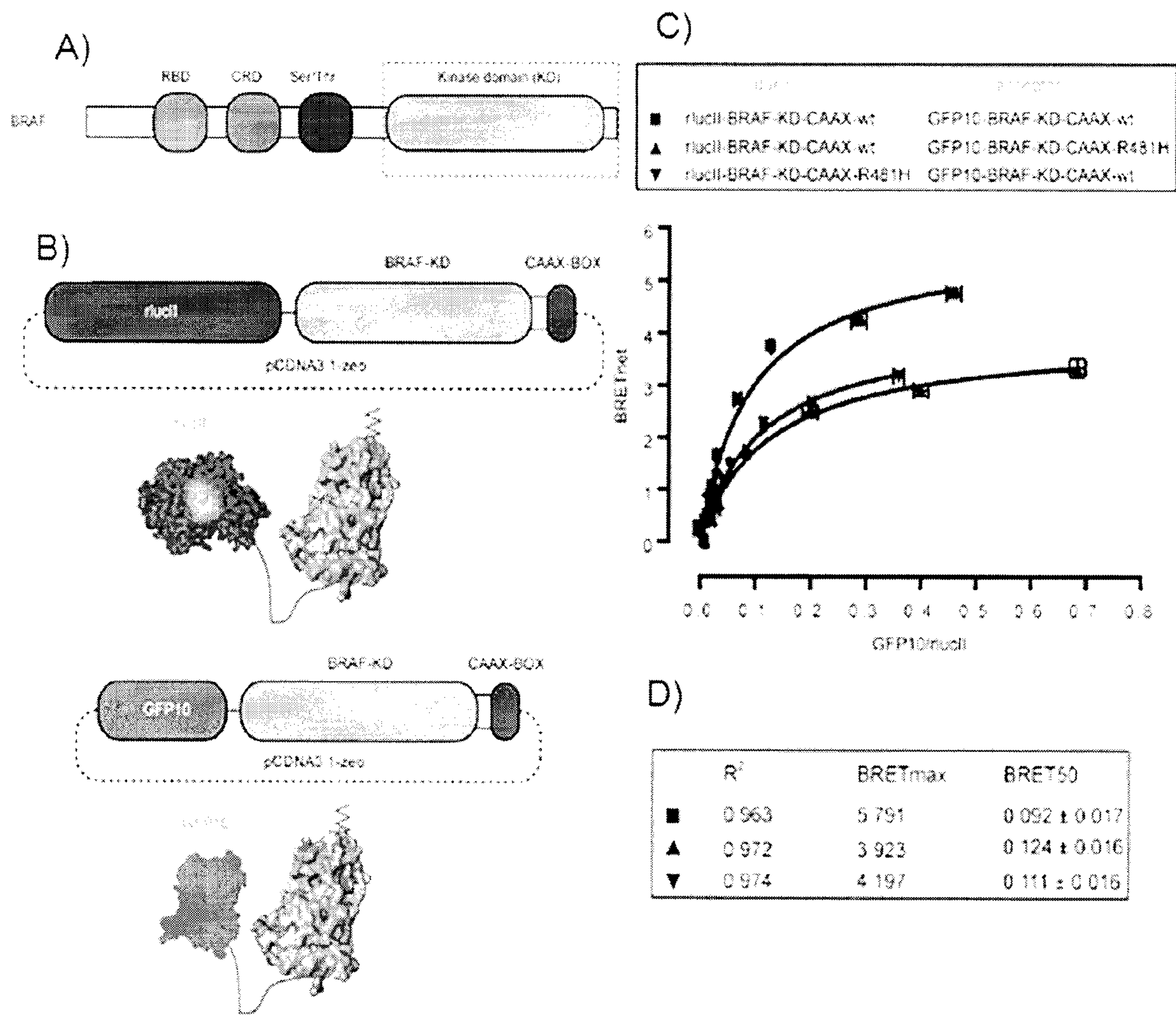


Figure 15

CAAX-box and BRET donor and acceptor sequences

```
>human KRAS CAAX-box CDS
AAGATGAGCAAAGATGGTAAAAAGAAGAAAAAGAAGTCAAAGACAAAGTGTGTAATTA
TGTA (SEQ ID NO. 19)
>human KRAS CAAX-box
KMSKDGKKKKKKSKTKCVIM (SEQ ID NO. 20)
>GFP10 CDS
ATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTCGAGCTGG
ACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCAC
CTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCCGTGCCCTGG
CCCACCCTCGTGACCACCCTGAGCTACGGCGTGCTTCAGCCGCTACCCCGACC
ACATGAAGCAGCAGACTTCTTCAAGTCCGCCATGCCCCGAAGGCTACGTCCAGGAGCG
CACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAG
GGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCA
ACATCCTGGGGCACAAGCTGGAGTACAACCAACCAACCAACCAACCAACCAACCAACCA
CGACAAGCAGAAGAACGGCATCAAGGTGAAGTTCAGATCCGCCACAACATCGAGGAC
GGCAGCGTGAGCTCGCCGACCACTACCAGCAGAACACCCCATCGGCGACGGCCCCG
TGCTGCTGCCCCGACAACCACTACCTGTTACCCAGTCCGCCCTGAGCAAAGACCCCAA
CGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCGGGATCACTCTC
GGCATGGACGAGCTGTACAAGGATCCGCCTAG (SEQ ID NO. 21)
>GFP10
MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLLKFICTTGKLPVPW
PTLVTTLSYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAQEVKFE
GDTLVNRIELKGIDFKEDGNILGHKLEYNYNPHNVYIMADKQKNGIKVNFKIRHNIED
GSVQLADHYQQNTPIGDGPVLLPDNHYLEFTQSALSKDPNEKRDHMLLEFVTAAGITL
GMDELYKGS (SEQ ID NO. 22)
```

Figure 16

>rlucII CDS

ATGACCAGCAAGGTGTACGACCCCGAGCAGAGGAAGAGGATGATCACCGGCCCCCAGT
GGTGGGCCAGGTGCAAGCAGATGAACGTGCTGGACAGCTTCATCAACTACTACGACAG
CGAGAAGCACGCCGAGAACGCCGTGATCTTCCTGCACGGCAACGCCACTAGCAGCTAC
CTGTGGAGGCACGTGGTGCCCCACATCGAGCCCGTGGCCAGGTGCATCATCCCCGATC
TGATCGGCATGGGCAAGAGCGGCAAGAGCGGCAACGGCAGCTACAGGCTGCTGGACCA
CTACAAGTACCTGACCGCCTGGTTCGAGCTCCTGAACCTGCCCAAGAAGATCATCTTC
GTGGGCCACGACTGGGGCGCCGCACTGGCCTTCCACTACAGCTACGAGCACCAGGACA
AGATCAAGGCCATCGTGACGCGCCGAGAGCGTGGTTGACGTGATCGAGAGCTGGGACGA
GTGGCCAGACATCGAGGAGGACATCGCCCTGATCAAGAGCGAGGAGGGCGAGAAGATG
GTGCTGGAGAACAACCTTCTTCGTGGAGACCGTTCTGCCCAGCAAGATCATGAGAAAGC
TGGAGCCCGAGGAGTTCGCCGCCTACCTGGAGCCCTTCAAGGAGAAGGGCGAGGTGAG
AAGACCCACCCTGAGCTGGCCCAGAGAGATCCCCCTGGTGAAGGGCGGCAAGCCCGAC
GTGGTGCAGATCGTGAGAACTACAACGCCTACCTGAGAGCCAGCGACGACCTGCCCA
AGATGTTTCATCGAGAGCGACCCCGGCTTCTTCAGCAACGCCATCGTGGAGGGCGCCAA
GAAGTTCCCCAACACCGAGTTCGTGAAGGTGAAGGGCCTGCACTTCAGCCAGGAGGAC
GCCCCCGACGAGATGGGCAAGTACATCAAGAGCTTCGTGGAGAGAGTGCTGAAGAACG
AGCAGggatccgcctag (SEQ ID NO. 23)

>rlucII

MTSKVYDPEQRKRMITGPQWWARCKQMNVLDSFINYYDSEKHAENAVIFLHGNATSSY
LWRHVVPHIEPVARCIIPDLIGMGKSGKSGNGSYRLLDHYKYLTAWFELNLPKKIIF
VGHDWGAALAFHYSYEHQDKIKAIVHAESVVDVIESWDEWPDIEEDIALIKSEEKEM
VLENNFFVETVLP SKIMRKLEPEEFAAYLEPFKEKGEVRRPTLSWPREIPLVKGGKPD
VVQIVRNYNAYLRASDDLPMFIESDPGFFSNAIVEGAKKFPNTEFVKVKGLHFSQED
APDEMGKYIKSFVERVLKNEQGS (SEQ ID NO. 24)

Figure 16 cont.

Human BRAF (hBRAF) sequences

>hBRAF-KD-wt CDS

ATGGATGATTGGGAGATTCCTGATGGGCAGATTACAGTGGGACAAAGAATTGGATCTG
GATCATTTGGAACAGTCTACAAGGGAAAGTGGCATGGTGTGATGTGGCAGTGAAAATGTT
GAATGTGACAGCACCTACACCTCAGCAGTTACAAGCCTTCAAAAATGAAGTAGGAGTA
CTCAGGAAAACACGACATGTGAATATCCTACTCTTCATGGGCTATTCCACAAAGCCAC
AACTGGCTATTGTTACCCAGTGGTGTGAGGGCTCCAGCTTGTATCACCATCTCCATAT
CATTGAGACCAAATTTGAGATGATCAAACCTTATAGATATTGCACGACAGACTGCACAG
GGCATGGATTACTTACACGCCAAGTCAATCATCCACAGAGACCTCAAGAGTAATAATA
TATTTCTTCATGAAGACCTCACAGTAAAAATAGGTGATTTTGGTCTAGCTACAGTGAA
ATCTCGATGGAGTGGGTCCCATCAGTTTGAACAGTTGTCTGGATCCATTTTGTGGATG
GCACCAGAAGTCATCAGAATGCAAGATAAAAATCCATACAGCTTTCAGTCAGATGTAT
ATGCATTTGGAATTGTTCTGTATGAATTGATGACTGGACAGTTACCTTATTCAAACAT
CAACAACAGGGACCAGATAATTTTTATGGTGGGACGAGGATACCTGTCTCCAGATCTC
AGTAAGGTACGGAGTAACTGTCCAAAAGCCATGAAGAGATTAATGGCAGAGTGCCTCA
AAAAGAAAAGAGATGAGAGACCACTCTTTCCCCAAATTCTCGCCTCTATTGAGCTGCT
GGCCCGCTCATTGCCAAAATTACCCGCAGTGCATCAGAACCCTCCTTGAATCGGGCT
GGTTTCCAAACAGAGGATTTTAGTCTATATGCTTGTGCTTCTCCAAAACACCCATCC
AGGCAGGGGGATATGGTGCGTTTCCTGTCCAC (SEQ ID NO. 25)

>hBRAF-KD-wt

MDDWEIPDGQITVGQRIGSGSFGTVYKGKWHGDVAVKMLNVTAPTQQQLQAFKNEVGV
LRKTRHVNILLFMGYSTKPQLAIVTQWCEGSSLYHHLHIIETKFEMIKLIDIARQTAQ
GMDYLHAKSIIHRDLKSNNIFLHEDLTVKIGDFGLATVKSRWSGSHQFEQLSGSILWM
APEVIRMQDKNPYSFQSDVYAFGIVLYELMTGQLPYSNINNRDQIIIFMVGRGYLSPDL
SKVRSNCPKAMKRLMAECLKKKRDERPLFPQILASIELLARS LPKIHRSASEPSLNRA
GFQTEDFS LYACASPKTPIQAGGYGAFPVH (SEQ ID NO. 26)

Figure 17

>hBRAF-KD-R481H CDS

ATGGATGATTGGGAGATTCCTGATGGGCAGATTACAGTGGGACAAAGAATTGGATCTG
GATCATTTGGAACAGTCTACAAGGGAAAGTGGCATGGTGATGTGGCAGTGAAAATGTT
GAATGTGACAGCACCTACACCTCAGCAGTTACAAGCCTTCAAAAATGAAGTAGGAGTA
CTCAGGAAAACA**CAT**CATGTGAATATCCTACTCTTCATGGGCTATTCCACAAAGCCAC
AACTGGCTATTGTTACCCAGTGGTGTGAGGGGCTCCAGCTTGTATCACCATCTCCATAT
CATTGAGACCAAATTTGAGATGATCAAACCTTATAGATATTGCACGACAGACTGCACAG
GGCATGGATTACTTACACGCCAAGTCAATCATCCACAGAGACCTCAAGAGTAATAATA
TATTTCTTCATGAAGACCTCACAGTAAAAATAGGTGATTTTGGTCTAGCTACAGTGAA
ATCTCGATGGAGTGGGTCCCATCAGTTTGAACAGTTGTCTGGATCCATTTTGTGGATG
GCACCAGAAGTCATCAGAATGCAAGATAAAAATCCATACAGCTTTCAGTCAGATGTAT
ATGCATTTGGAATTGTTCTGTATGAATTGATGACTGGACAGTTACCTTATTCAAACAT
CAACAACAGGGACCAGATAATTTTTATGGTGGGACGAGGATACCTGTCTCCAGATCTC
AGTAAGGTACGGAGTAACTGTCCAAAAGCCATGAAGAGATTAATGGCAGAGTGCCTCA
AAAAGAAAAGAGATGAGAGACCACTCTTTCCCAAATTCTCGCCTCTATTGAGCTGCT
GGCCCGCTCATTGCCAAAATTCACCGCAGTGCATCAGAACCCTCCTTGAATCGGGCT
GGTTTCCAAACAGAGGATTTTAGTCTATATGCTTGTGCTTCTCCAAAACACCCATCC
AGGCAGGGGGATATGGTGC GTTTCCTGTCCAC (SEQ ID NO. 27)

>hBRAF-KD-R481H

MDDWEIPDGQITVGQRIGSGSFGTVYKGKWHGDVAVKMLNVTAPTQQQLQAFKNEVGV
LRKT**H**HVNILLFMGYSTKPQLAIVTQWCEGSSLYHHLHIIETKFEMIKLIDIARQTAQ
GMDYLHAKSIIHRDLKSNNIFLHEDLTVKIGDFGLATVKSRSWGS HQFEQLSGSILWM
APEVIRMQDKNPYSFQSDVYAFGIVLYELMTGQLPYSNINNRDQIIIFMVGRGYLSPDL
SKVRSNCPKAMKRLMAECLKKRDERPLFPQILASIELLARS LPKIHR SASEPSLNRA
GFQTEDFS LYACASPKTPIQAGGYGAFPVH (SEQ ID NO. 28)

Figure 17 cont.

All human BRAF-KD clones are between the NheI and XbaI sites in pCDNA3.1-zeo

>GFP10-hBRAF-KD-wt-CAAX CDS

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGG
ACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCAC
CTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCCGTGCCCTGG
CCCACCCTCGTGACCACCCTGAGCTACGGCGTGCAAGTTCAGCCGCTACCCCGACC
ACATGAAGCAGCAGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCG
CACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCCGCGCCGAGGTGAAGTTCGAG
GGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCA
ACATCCTGGGGCACAAGCTGGAGTACAACCTACAACCCCCACAACGTCTATATCATGGC
CGACAAGCAGAAGAACGGCATCAAGGTGAAGTTCAGATCCGCCACAACATCGAGGAC
GGCAGCGTGCAAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCG
TGCTGCTGCCCCGACAACCACTACCTGTTCACCCAGTCCGCCCTGAGCAAAGACCCCAA
CGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTC
GGCATGGACGAGCTGTACAAGGGATCCGCCGGTACCATGGATGATTGGGAGATTCTTG
ATGGGCAGATTACAGTGGGACAAAGAATTGGATCTGGATCATTGGAACAGTCTACAA
GGGAAAGTGGCATGGTGTATGTGGCAGTGAAATGTTGAATGTGACAGCACCTACACCT
CAGCAGTTACAAGCCTTCAAAAATGAAGTAGGAGTACTCAGGAAAACACGACATGTGA
ATATCCTACTCTTCATGGGCTATTCCACAAAGCCACAACCTGGCTATTGTTACCCAGTG
GTGTGAGGGCTCCAGCTTGTATCACCATCTCCATATCATTGAGACCAAATTTGAGATG
ATCAAACCTTATAGATATTGCACGACAGACTGCACAGGGCATGGATTACTTACACGCCA
AGTCAATCATCCACAGAGACCTCAAGAGTAATAATATATTTCTTCATGAAGACCTCAC
AGTAAAAATAGGTGATTTTGGTCTAGCTACAGTGAAATCTCGATGGAGTGGGTCCCAT
CAGTTTGAACAGTTGTCTGGATCCATTTTGTGGATGGCACCAGAAGTCATCAGAATGC
AAGATAAAAATCCATACAGCTTTCAGTCAGATGTATATGCATTTGGAATTGTTCTGTA
TGAATTGATGACTGGACAGTTACCTTATTCAAACATCAACAACAGGGACCAGATAATT
TTTATGGTGGGACGAGGATACCTGTCTCCAGATCTCAGTAAGGTACGGAGTAACTGTC
CAAAAGCCATGAAGAGATTAATGGCAGAGTGCCTCAAAAAGAAAAGAGATGAGAGACC
ACTCTTTCCCAAATTTCTCGCCTCTATTGAGCTGCTGGCCCGCTCATTGCCAAAATTT
CACCGCAGTGCATCAGAACCCTCCTTGAATCGGGCTGGTTTCCAAACAGAGGATTTTA
GTCTATATGCTTGTGCTTCTCCAAAACACCCATCCAGGCAGGGGGATATGGTGCGTT
TCCTGTCCACAAGATGAGCAAAGATGGTAAAAAGAAGAAAAGAAGTCAAAGACAAAG
TGTGTAATTATGTAA (SEQ ID NO. 29)

Figure 18

>GFP10-hBRAF-KD-wt-CAAX

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTCLKFICTTGKLPVPW
PTLVTTLSYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFE
GDTLVNRIELKGIDFKEDGNILGHKLEYNYNPHNVYIMADKQKNGIKVNFKIRHNIED
GSVQLADHYQQNTPIGDGPVLLPDNHYLFTQSALSKDPNEKRDHMLLEFVTAAGITL
GMDELYK**GSAGT**MDDWEIPDGQITVGQRIGSGSFGTVYKKGKWHGDVAVKMLNVTAPTP
QQQLQAFKNEVGVLRLKTRHVNILLFMGYSTKPQLAIVTQWCEGSSLYHHLHIIETKFEM
IKLIDDIARQTAQGM DY LHAKSIIHRDLKSNNIFLHEDLTVKIGDFGLATVKSRWSGSH
QFEQLSGSILWMAPEVIRMQDKNPYSFQSDVYAFGIVLYELMTGQLPYSNINN RDQII
FMVGRGYLSPDLSKVRSNCPKAMKRLMAECLKKKRDERPLFPQILASIELLARSLPKI
HRSASEPSLNRAGFQTEDEFSLYACASPKTPIQAGGYGAFPVHKMSKDGKKKKKKSKTK
CVIM (SEQ ID NO. 30)

>GFP10-hBRAF-KD-R481H-CAAX CDS

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ACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCAC
CTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGG
CCCACCCTCGTGACCACCCTGAGCTACGGCGTGCGAGTGCTTCAGCCGCTACCCCGACC
ACATGAAGCAGCAGCACTTCTTCAAGTCCGCCATGCCCCGAAGGCTACGTCCAGGAGCG
CACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAG
GGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCA
ACATCCTGGGGCACAAGCTGGAGTACAAC TACAACCCCCACAACGTCTATATCATGGC
CGACAAGCAGAAGAACGGCATCAAGGTGAAC TCAAGATCCGCCACAACATCGAGGAC
GGCAGCGTGCGAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCG
TGCTGCTGCCCCGACAACCACTACCTGTTCACCCAGTCCGCCCTGAGCAAAGACCCCAA
CGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTC
GGCATGGACGAGCTGTACAAG**GGATCCGCCGGTACC**ATGGATGATTGGGAGATTCTTG
ATGGGCAGATTACAGTGGGACAAAGAATTGGATCTGGATCATTTGGAACAGTCTACAA
GGGAAAGTGGCATGGTGATGTGGCAGTGAAAATGTTGAATGTGACAGCACCTACACCT
CAGCAGTTACAAGCCTTCAAAAATGAAGTAGGAGTACTCAGGAAAACA**CAT**CATGTGA
ATATCCTACTCTTCATGGGCTATTCCACAAAGCCACAAC TGGCTATTGTTACCCAGTG
GTGTGAGGGCTCCAGCTTGTATCACCATCTCCATATCATTGAGACCAAATTTGAGATG
ATCAAAC TTAGATATTGCACGACAGACTGCACAGGGCATGGATTACTTACACGCCA
AGTCAATCATCCACAGAGACCTCAAGAGTAATAATATATTTCTTCATGAAGACCTCAC
AGTAAAAATAGGTGATTTTGGTCTAGCTACAGTGAAATCTCGATGGAGTGGGTCCCAT
CAGTTTGAACAGTTGTCTGGATCCATTTTGTGGATGGCACCAGAAGTCATCAGAATGC
AAGATAAAAATCCATACAGCTTTCAGTCAGATGTATATGCATTTGGAATTGTTCTGTA
TGAATTGATGACTGGACAGTTACCTTATTCAAACATCAACAACAGGGACCAGATAATT
TTTATGGTGGGACGAGGATACCTGTCTCCAGATCTCAGTAAGGTACGGAGTAACTGTC
CAAAAGCCATGAAGAGATTAATGGCAGAGTGCCTCAAAAAGAAAAGAGATGAGAGACC
ACTCTTTCCCCAAATTCTCGCCTCTATTGAGCTGCTGGCCCGCTCATTGCCAAAAATT
CACCGCAGTGCATCAGAACCCTCCTTGAATCGGGCTGGTTTCCAAACAGAGGATTTTA
GTCTATATGCTTGTGCTTCTCCAAAAACACCCATCCAGGCAGGGGGATATGGTGCGTT
TCCTGTCCACAAGATGAGCAAAGATGGTAAAAAGAAGAAAAGAAGTCAAAGACAAAG
TGTGTAATTATGTAA (SEQ ID NO. 31)

Figure 18 cont.

>GFP10-hBRAF-KD-R481H-CAAX
 MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTCLKFICTTGKLPVPW
 PTLVTTLSYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFE
 GDTLVNRIELKGIDFKEDGNILGHKLEYNYPHNVIYIMADKQKNGIKVNFKIRHNIED
 GSVQLADHYQQNTPIGDGPVLLPDNHYLEFTQSALS KDPNEKRDHMLLEFVTAAGITL
 GMDELYK**GSAGT**MDDWEIPDGQITVGQRIGSGSFGTVYKGKWHGDVAVKMLNVTAPT
 QQLQAFKNEVGVLARKT**H**HVNILLFMGYSTKPQLAIVTQWCEGSSLYHHLHIETKFEM
 IKLIDIARQTAQGMDYLHAKSIIHRDLKSNNIFLHEDLTVKIGDFGLATVKSRWSGSH
 QFEQLSGSILWMAPEVIRMQDKNPYSFQSDVYAFGIVLYELMTGQLPYSNINNDRDQII
 FMVGRGYLSPDLKSVRSNCPKAMKRLMAECLKKKRDERPLFPQILASIELLARS LPKI
 HRSASEPSLN RAGFQTEDFS LYACASPKTPIQAGGYGAFFVHKMSKDGK KKKKKKSKTK
 CVIM (SEQ ID NO. 32)

>rlucII-hBRAF-KD-wt-CAAX CDS
 ATGACCAGCAAGGTGTACGACCCCGAGCAGAGGAAGAGGATGATCACCGGCCCCCAGT
 GGTGGGCCAGGTGCAAGCAGATGAACGTGCTGGACAGCTTCATCAACTACTACGACAG
 CGAGAAGCACGCCGAGAACGCCGTGATCTTCCTGCACGGCAACGCCACTAGCAGCTAC
 CTGTGGAGGCACGTGGTGCCCCACATCGAGCCCGTGGCCAGGTGCATCATCCCCGATC
 TGATCGGCATGGGCAAGAGCGGCAAGAGCGGCAACGGCAGCTACAGGCTGCTGGACCA
 CTACAAGTACCTGACCGCCTGGTTCGAGCTCCTGAACCTGCCCAAGAAGATCATCTTC
 GTGGGCCACGACTGGGGCGCCGCACTGGCCTTCCACTACAGCTACGAGCACCAGGACA
 AGATCAAGGCCATCGTGACGCCGAGAGCGTGGTTGACGTGATCGAGAGCTGGGACGA
 GTGGCCAGACATCGAGGAGGACATCGCCCTGATCAAGAGCGAGGAGGGCGAGAAGATG
 GTGCTGGAGAACAACCTTCTTCGTGGAGACCGTTCTGCCCAGCAAGATCATGAGAAAGC
 TGGAGCCCGAGGAGTTCGCCGCCTACCTGGAGCCCTTCAAGGAGAAGGGCGAGGTGAG
 AAGACCCACCCTGAGCTGGCCAGAGAGATCCCCCTGGTGAAGGGCGGCAAGCCCGAC
 GTGGTGCAGATCGTGAGAACTACAACGCCTACCTGAGAGCCAGCGACGACCTGCCCA
 AGATGTTTCATCGAGAGCGACCCCGGCTTCTTCAGCAACGCCATCGTGGAGGGCGCCAA
 GAAGTTCCCCAACACCGAGTTCGTGAAGGTGAAGGGCCTGCACTTCAGCCAGGAGGAC
 GCCCCCGACGAGATGGGCAAGTACATCAAGAGCTTCGTGGAGAGAGTGCTGAAGAACG
 AGCAG**ggatccggcGGTACC**ATGGATGATTGGGAGATTCCTGATGGGCAGATTACAGT
 GGGACAAAGAATTGGATCTGGATCATTTGGAACAGTCTACAAGGGAAAGTGGCATGGT
 GATGTGGCAGTGAAAATGTTGAATGTGACAGCACCTACACCTCAGCAGTTACAAGCCT
 TCAAAAATGAAGTAGGAGTACTCAGGAAAACACGACATGTGAATATCCTACTCTTCAT
 GGGCTATTCCACAAAGCCACAACCTGGCTATTGTTACCCAGTGGTGTGAGGGCTCCAGC
 TTGTATCACCATCTCCATATCATTGAGACCAAATTTGAGATGATCAAACCTTATAGATA
 TTGCACGACAGACTGCACAGGGCATGGATTA CTTACACGCCAAGTCAATCATCCACAG
 AGACCTCAAGAGTAATAATATATTTCTTCATGAAGACCTCACAGTAAAAATAGGTGAT
 TTTGGTCTAGCTACAGTGAAATCTCGATGGAGTGGGTCCCATCAGTTTGAACAGTTGT
 CTGGATCCATTTTGTGGATGGCACCAGAAAGTCATCAGAATGCAAGATAAAAATCCATA
 CAGCTTTCAGTCAGATGTATATGCATTTGGAATTGTTCTGTATGAATTGATGACTGGA
 CAGTTACCTTATTCAAACATCAACAACAGGGACCAGATAATTTTTATGGTGGGACGAG
 GATACCTGTCTCCAGATCTCAGTAAGGTACGGAGTAACTGTCCAAAAGCCATGAAGAG
 ATTAATGGCAGAGTGCCTCAAAAAGAAAAGAGATGAGAGACCACTCTTTCCCCAAATT
 CTCGCCTCTATTGAGCTGCTGGCCCGCTCATTTGCCAAAATTCACCGCAGTGCATCAG
 AACCTCCTTGAATCGGGCTGGTTTCCAAACAGAGGATTTT TAGTCTATATGCTTGTGC
 TTCTCCAAAACACCCATCCAGGCAGGGGGATATGGTGCGTTTCTGTCCACAAGATG
 AGCAAAGATGGTAAAAAGAAGAAAAGAAGTCAAAGACAAAGTGTGTAATTATGTAA
 (SEQ ID NO. 33)

Figure 18 cont.

>rlucII-hBRAF-KD-wt-CAAX

MTSKVYDPEQRKRMITGPQWWARCKQMNVLDSFINYYDSEKHAENAVIFLHGNATSSY
LWRHVVPHIEPVARCIIPDLIGMGKSGKSGNGSYRLLDHYKYLTAWFELNLPKKIIF
VGHDWGAALAFHYSYEHQDKIKAIVHAESVVDVIESWDEWPDIEEDIALIKSEEGEKM
VLENNFFVETVLP SKIMRKLEPEEFAAYLEPFKEKGEVRRPTLSWPREIPLVKGGKPD
VVQIVRNYNAYLRASDDL PKMFIESDPGFFSNAIVEGAKKFPNTEFVKVKGLHFSQED
APDEMGKYIKSFVERVLKNEQ**GSAGT**MDDWEIPDGQITVGQRIGSGSFGTVYKGKWHG
DVAVKMLNVTAPTQQQLQAFKNEVGVL RKTRHVNILLFMGYSTKPQLAIVTQWCEGSS
LYHHLHIETKFEMIKLIDIA RQTAQGMDYLHAKSIIHRDLKSNNIFLHEDLTVKIGD
FGLATVKSRWSGSHQFEQLSGSILWMAPEVIRMQDKNPYSFQSDVYA FGI VLYELMTG
QLPYSNINN RDQIIFMVGRGYLSPDLSKVR SNCPKAMKRLMAECLKKRDERPLFPQI
LASIELLARS LPKIHRSASEPSLNRAGFQTEDFS LYACASPKTPIQAGGYGAFPVHKM
SKDGKKKKKKSKTKCVIM (SEQ ID NO. 34)

Figure 18 cont.

>rlucII-hBRAF-KD-R481H-CAAX CDS
ATGACCAGCAAGGTGTACGACCCCGAGCAGAGGAAGAGGATGATCACCGGCCCCCAGT
GGTGGGCCAGGTGCAAGCAGATGAACGTGCTGGACAGCTTCATCAACTACTACGACAG
CGAGAAGCACGCCGAGAACGCCGTGATCTTCCTGCACGGCAACGCCACTAGCAGCTAC
CTGTGGAGGCACGTGGTGCCCCACATCGAGCCCGTGGCCAGGTGCATCATCCCCGATC
TGATCGGCATGGGCAAGAGCGGCAAGAGCGGCAACGGCAGCTACAGGCTGCTGGACCA
CTACAAGTACCTGACCGCCTGGTTCGAGCTCCTGAACCTGCCCCAAGAAGATCATCTTC
GTGGGCCACGACTGGGGCGCCGCACTGGCCTTCCACTACAGCTACGAGCACCAGGACA
AGATCAAGGCCATCGTGCACGCCGAGAGCGTGGTTGACGTGATCGAGAGCTGGGACGA
GTGGCCAGACATCGAGGAGGACATCGCCCTGATCAAGAGCGAGGAGGGCGAGAAGATG
GTGCTGGAGAACAACCTTCTTCGTGGAGACCGTTCTGCCAGCAAGATCATGAGAAAGC
TGGAGCCCGAGGAGTTCGCCGCCTACCTGGAGCCCTTCAAGGAGAAGGGCGAGGTGAG
AAGACCCACCCTGAGCTGGCCCAGAGAGATCCCCCTGGTGAAGGGCGGCAAGCCCGAC
GTGGTGCAGATCGTGAGAACTACAACGCCTACCTGAGAGCCAGCGACGACCTGCCCA
AGATGTTTCATCGAGAGCGACCCCGGCTTCTTCAGCAACGCCATCGTGGAGGGCGCCAA
GAAGTTCCCCAACACCGAGTTCGTGAAGGTGAAGGGCCTGCACTTCAGCCAGGAGGAC
GCCCCCGACGAGATGGGCAAGTACATCAAGAGCTTCGTGGAGAGAGTGCTGAAGAACG
AGCAG**ggatccgccGGTACC**ATGGATGATTGGGAGATTCCTGATGGGCAGATTACAGT
GGGACAAAGAATTGGATCTGGATCATTTGGAACAGTCTACAAGGGAAAGTGGCATGGT
GATGTGGCAGTGAAAATGTTGAATGTGACAGCACCTACACCTCAGCAGTTACAAGCCT
TCAAAAATGAAGTAGGAGTACTCAGGAAAACA**CAT**CATGTGAATATCCTACTCTTCAT
GGGCTATTCCACAAAGCCACAACCTGGCTATTGTTACCCAGTGGTGTGAGGGCTCCAGC
TTGTATCACCATCTCCATATCATTGAGACCAAATTTGAGATGATCAAACCTTATAGATA
TTGCACGACAGACTGCACAGGGCATGGATTACTTACACGCCAAGTCAATCATCCACAG
AGACCTCAAGAGTAATAATATATTTCTTCATGAAGACCTCACAGTAAAAATAGGTGAT
TTTGGTCTAGCTACAGTGAAATCTCGATGGAGTGGGTCCCATCAGTTTGAACAGTTGT
CTGGATCCATTTTGTGGATGGCACCAGAAGTCATCAGAATGCAAGATAAAAAATCCATA
CAGCTTTCAGTCAGATGTATATGCATTTGGAATTGTTCTGTATGAATTGATGACTGGA
CAGTTACCTTATTCAAACATCAACAACAGGGACCAGATAATTTTATGGTGGGACGAG
GATACCTGTCTCCAGATCTCAGTAAGGTACGGAGTAACTGTCCAAAAGCCATGAAGAG
ATTAATGGCAGAGTGCCTCAAAAAGAAAAGAGATGAGAGACCACTCTTTCCCCAAATT
CTCGCCTCTATTGAGCTGCTGGCCCGCTCATTGCCAAAAATTACCGCAGTGCATCAG
AACCTCCTTGAATCGGGCTGGTTTCCAA
ACAGAGGATTTTAGTCTATATGCTTGTGCTTCTCCAAAAACACCCATCCAGGCAGGGG
GATATGGTGCGTTTCCTGTCCACAAGATGAGCAAAGATGGTAAAAAGAAGAAAAAGAA
GTCAAAGACAAAGTGTGTAATTATGTAA (SEQ ID NO. 35)

Figure 18 cont.

>rlucII-hBRAF-KD-R481H-CAAX

MTSKVYDPEQRKRMITGPQWWARCKQMNVLDSFINYYDSEKHAENAVIFLHGNATSSY
LWRHVVPPIEPVARCIIPDLIGMGKSGKSGNGSYRLLDHYKYLTAWFELNLPKKII F
VGHDWGAALAFHYSYEHQDKIKAIVHAESVVDVIESWDEWPDIEEDIALIKSEEGEKM
VLENNFFVETVLP SKIMRKLEPEEFAAYLEPFKEKGEVRRPTLSWPREIPLVKGGKPD
VVQIVRNYNAYLRASDDL PKMFIESDPGFFSNAIVEGAKKFPNTEFVKVKGLHFSQED
APDEMGKYIKSFVERVLKNEQ**GSAGT**MDDWEIPDGQITVGQRIGSGSF GTVYKGKWHG
DVAVKMLNVTAPT PQQLQAFKNEVGVL RKT**H**VNILLFMGYSTKPQLAIVTQWCEGSS
LYHHLHI IETKFEMI KLIDIARQTAQGMDYLHAKSIIHRDLKSNNIFLHEDLTVKIGD
FGLATVKSRWSGSHQFEQLSGSILWMAPEVIRMQDKNPYSFQSDVYA FGI VLYELMTG
QLPYSNINN RDQII FMVGRGYLSPDLSKVR SNCPKAMKRLMAECLKKKRDERPLFPQI
LASIELLARSLPKIHR SASEPSLNRAGFQTEDFS LYACASPKTPIQAGGYGAFPVHKM
SKDGKKKKKKSKTKCVIM (SEQ ID NO. 36)

Figure 18 cont.

Human CRAF (hCRAF) sequences

>hCRAF-KD-wt CDS

ATGTATTATTGGGAAATAGAAGCCAGTGAAGTGATGCTGTCCACTCGGATTGGGTCAG
GCTCTTTTGGAACTGTTTATAAGGGTAAATGGCACGGAGATGTTGCAGTAAAGATCCT
AAAGGTTGTCGACCCAACCCCAAGCAATTCCAGGCCTTCAGGAATGAGGTGGCTGTT
CTGCGCAAAACACGGCATGTGAACATTCTGCTTTTCATGGGGTACATGACAAAGGACA
ACCTGGCAATTGTGACCCAGTGGTGCGAGGGCAGCAGCCTCTACAAACACCTGCATGT
CCAGGAGACCAAGTTTCAGATGTTCCAGCTAATTGACATTGCCCCGGCAGACGGCTCAG
GGAATGGACTATTTGCATGCAAAGAACATCATCCATAGAGACATGAAATCCAACAATA
TATTTCTCCATGAAGGCTTAACAGTGAAAATTGGAGATTTTGGTTTGGCAACAGTAAA
GTCACGCTGGAGTGGTTCTCAGCAGGTTGAACAACCTACTGGCTCTGTCCTCTGGATG
GCCCCAGAGGTGATCCGAATGCAGGATAACAACCCATTCAGTTTCCAGTCGGATGTCT
ACTCCTATGGCATCGTATTGTATGAACTGATGACGGGGGAGCTTCCTTATTCTCACAT
CAACAACCGAGATCAGATCATCTTCATGGTGGGCGGAGGATATGCCTCCCCAGATCTT
AGTAAGCTATATAAGAACTGCCCCAAAGCAATGAAGAGGCTGGTAGCTGACTGTGTGA
AGAAAGTAAAGGAAGAGAGGCCTCTTTTTCCCCAGATCCTGTCTTCCATTGAGCTGCT
CCAACACTCTCTACCGAAGATCAACCGGAGCGCTTCCGAGCCATCCTTGCATCGGGCA
GCCCACACTGAGGATATCAATGCTTGCACGCTGACCACGTCCCCGAGGCTGCCTGTCT
TCTAG (SEQ ID NO. 37)

>hCRAF-KD-wt

MYWEIEASEVMLSTRIGSGSFGTVYKGKWHGDVAVKILKVVDPTPEQFQAFRNEVAV
LRKTRHVNILLFMGYMTKDNLAIVTQWCEGSSLYKHLHVQETKFQMFQLIDIARQTAQ
GMDYLHAKNIIHRDMKSNNIFLHEGLTVKIGDFGLATVKSRSWSGSQQVEQPTGSVLWM
APEVIRMQDNNPFSFQSDVYSYGIVLYELMTGELPYSHINNDRDQIIFMVGRGYASPD
SKLYKNCPKAMKRLVADCVKKVKEERPLFPQILSSIELLQHSPLPKINRSASEPSLHRA
AHTEDINACTLTTSPLRPVF (SEQ ID NO. 38)

Figure 19

All hCRAF-KD fusions are cloned between NheI and XbaI in pCDNA3.1-zeo

>GFP10-hCRAF-KD-wt-CAAX CDS

ATGGTGAGCAAGGGGCGAGGAGCTGTTACCCGGGGTGGTGCCCATCCTGGTTCGAGCTGG
ACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCAC
CTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCCGTGCCCTGG
CCCACCCTCGTGACCACCCTGAGCTACGGCGTGCAAGTCTCAGCCGCTACCCCGACC
ACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCCGAAGGCTACGTCCAGGAGCG
CACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAG
GGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCA
ACATCCTGGGGCACAAGCTGGAGTACAACCTACAACCCCCACAACGTCTATATCATGGC
CGACAAGCAGAAGAACGGCATCAAGGTGAAGTTCAGATCCGCCACAACATCGAGGAC
GGCAGCGTGCAAGCTCGCCGACCACTACCAGCAGAACACCCCATCGGCGACGGCCCCG
TGCTGCTGCCCCGACAACCACTACCTGTTACCCAGTCCGCCCTGAGCAAAGACCCCAA
CGAGAAGCGCGATCACATGGTCTGCTGGAGTTCGTGACCGCCGCGGGGATCACTCTC
GGCATGGACGAGCTGTACAAG**GGATCCGCCGGTACC**ATGTATTATTGGGAAATAGAAG
CCAGTGAAGTGATGCTGTCCACTCGGATTGGGTTCAGGCTCTTTTGGAAGTGTATATAA
GGGTAAATGGCACGGAGATGTTGCAGTAAAGATCCTAAAGGTTGTCGACCCAACCCCA
GAGCAATTCCAGGCCTTCAGGAATGAGGTGGCTGTTCTGCGCAAAACACGGCATGTGA
ACATTCTGCTTTTCATGGGGTACATGACAAAGGACAACCTGGCAATTGTGACCCAGTG
GTGCGAGGGCAGCAGCCTCTACAAACACCTGCATGTCCAGGAGACCAAGTTTCAGATG
TTCCAGCTAATTGACATTGCCCCGGCAGACGGCTCAGGGAATGGACTATTTGCATGCAA
AGAACATCATCCATAGAGACATGAAATCCAACAATATATTTCTCCATGAAGGCTTAAC
AGTGAAAATTGGAGATTTTGGTTTGGCAACAGTAAAGTCACGCTGGAGTGGTTCTCAG
CAGGTTGAACAACCTACTGGCTCTGTCTCTGGATGGCCCCAGAGGTGATCCGAATGC
AGGATAACAACCCATTTCAGTTTCCAGTCGGATGTCTACTCCTATGGCATCGTATTGTA
TGAAGTATGACGGGGGAGCTTCCTTATTCTCACATCAACAACCGAGATCAGATCATC
TTCATGGTGGGCCGAGGATATGCCTCCCCAGATCTTAGTAAGCTATATAAGAACTGCC
CCAAAGCAATGAAGAGGCTGGTAGCTGACTGTGTGAAGAAAGTAAAGGAAGAGAGGCC
TCTTTTCCCCAGATCCTGTCTTCCATTGAGCTGCTCCAACACTCTCTACCGAAGATC
AACCGGAGCGCTTCCGAGCCATCCTTGCATCGGGCAGCCCACACTGAGGATATCAATG
CTTGCACGCTGACCACGTCCCCGAGGCTGCCTGTCTTCAAGATGAGCAAAGATGGTAA
AAAGAAGAAAAAGAAGTCAAAGACAAAGTGTGTAATTATGTAA (SEQ ID NO.
39)

>GFP10-hCRAF-KD-wt-CAAX

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLLKFICTTGKLPVPW
PTLVTTLSYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFE
GDTLVNRIELKGIDFKEDGNILGHKLEYNYNPHNVYIMADKQKNGIKVNFKIRHNIED
GSVQLADHYQQNTPIGDGPVLLPDNHYLEFTQSALSKDPNEKRDHMLLEFVTAAGITL
GMDELYK**GSAGT**MYWEIEASEVMLSTRIGSGSFQTVYKKGWHGDVAVKILKVVDPTP
EQFQAFRNEVAVLRKTRHVNILLFMGYMTKDNLAIIVTQWCEGSSLYKHLHVQETKFKQM
FQLIDIARQTAQGMDYLHAKNIIHRDMKSNNIFLHEGLTVKIGDFGLATVKSRWSGSQ
QVEQPTGSVLWMAPEVIRMQDNNPFSFQSDVYSYGIVLYELMTGELPYSHINNRDQII
FMVGRGYASPDLSKLYKNCPKAMKRLVADCVKKVKEERPLFPQILSSIPELLQHSPLPKI
NRSASEPSLHRAAHTEDINACTLTTSPLRPVFKMSKDGGKKKKKKSKTKCVIM (SEQ
ID NO. 40)

Figure 20

>rlucII-hCRAF-KD-wt-CAAX CDS
ATGACCAGCAAGGTGTACGACCCCGAGCAGAGGAAGAGGATGATCACCGGCCCCCAGT
GGTGGGCCAGGTGCAAGCAGATGAACGTGCTGGACAGCTTCATCAACTACTACGACAG
CGAGAAGCACGCCGAGAACGCCGTGATCTTCCTGCACGGCAACGCCACTAGCAGCTAC
CTGTGGAGGCACGTGGTGCCCCACATCGAGCCCGTGGCCAGGTGCATCATCCCCGATC
TGATCGGCATGGGCAAGAGCGGCAAGAGCGGCAACGGCAGCTACAGGCTGCTGGACCA
CTACAAGTACCTGACCGCCTGGTTTCGAGCTCCTGAACCTGCCCAAGAAGATCATCTTC
GTGGGCCACGACTGGGGCGCCGCACTGGCCTTCCACTACAGCTACGAGCACCAGGACA
AGATCAAGGCCATCGTGCACGCCGAGAGCGTGGTTGACGTGATCGAGAGCTGGGACGA
GTGGCCAGACATCGAGGAGGACATCGCCCTGATCAAGAGCGAGGAGGGCGAGAAGATG
GTGCTGGAGAACAACCTTCTTCGTGGAGACCGTTCTGCCCAGCAAGATCATGAGAAAGC
TGGAGCCCGAGGAGTTCGCCGCCTACCTGGAGCCCTTCAAGGAGAAGGGCGAGGTGAG
AAGACCCACCCTGAGCTGGCCCAGAGAGATCCCCCTGGTGAAGGGCGGCAAGCCCGAC
GTGGTGCAGATCGTGAGAACTACAACGCCTACCTGAGAGCCAGCGACGACCTGCCCA
AGATGTTTCATCGAGAGCGACCCCGGCTTCTTCAGCAACGCCATCGTGGAGGGCGCCAA
GAAGTTCCCCAACACCGAGTTCGTGAAGGTGAAGGGCCTGCACTTCAGCCAGGAGGAC
GCCCCCGACGAGATGGGCAAGTACATCAAGAGCTTCGTGGAGAGAGTGCTGAAGAACG
AGCAG**ggatccggcGGTACC**ATGTATTATTGGGAAATAGAAGCCAGTGAAGTGATGCT
GTCCACTCGGATTGGGTCAGGCTCTTTTGGAACTGTTTATAAGGGTAAATGGCACGGA
GATGTTGCAGTAAAGATCCTAAAGGTTGTCGACCCAACCCCGAGCAATTCCAGGCCT
TCAGGAATGAGGTGGCTGTTCTGCGCAAAACACGGCATGTGAACATTCTGCTTTTCAT
GGGGTACATGACAAAGGACAACCTGGCAATTGTGACCCAGTGGTGGCAGGGCAGCAGC
CTCTACAAACACCTGCATGTCCAGGAGACCAAGTTTCAGATGTTCCAGCTAATTGACA
TTGCCCCGGCAGACGGCTCAGGGAATGGACTATTTGCATGCAAAGAACATCATCCATAG
AGACATGAAATCCAACAATATATTTCTCCATGAAGGCTTAACAGTGAAAATTGGAGAT
TTTGGTTTGGCAACAGTAAAGTCACGCTGGAGTGGTTCTCAGCAGGTTGAACAACCTA
CTGGCTCTGTCTCTGGATGGCCCCAGAGGTGATCCGAATGCAGGATAACAACCCATT
CAGTTTCCAGTCGGATGTCTACTCCTATGGCATCGTATTGTATGAACTGATGACGGGG
GAGCTTCCTTATTCTCACATCAACAACCGAGATCAGATCATCTTCATGGTGGGCCGAG
GATATGCCTCCCCAGATCTTAGTAAGCTATATAAGAACTGCCCCAAAGCAATGAAGAG
GCTGGTAGCTGACTGTGTGAAGAAAGTAAAGGAAGAGAGGCCTCTTTTTCCCCAGATC
CTGTCTTCCATTGAGCTGCTCCAACACTCTCTACCGAAGATCAACCGGAGCGCTTCCG
AGCCATCCTTGCATCGGGCAGCCCACACTGAGGATATCAATGCTTGACGCTGACCAC
GTCCCCGAGGCTGCCTGTCTTCAAGATGAGCAAAGATGGTAAAAAGAAGAAAAAGAAG
TCAAAGACAAAGTGTGTAATTATGTAA (SEQ ID NO. 41)

Figure 20 cont.

>rlucII-hCRAF-KD-wt-CAAX
MTSKVYDPEQRKRMITGPQWWARCKQMNVLDSFINYYDSEKHAENAVIFLHGNATSSY
LWRHVVPPIEPVARCIIPDLIGMGKSGKSGNGSYRLLDHYKYLTAWFELNLPKKIIF
VGHDWGAALAFHYSYEHQDKIKAIVHAESVVDVIESWDEWPDIEEDIALIKSEEGEKM
VLENNFFVETVLP SKIMRKLEPEEFAAYLEPFKEKGEVRRPTLSWPREIPLVKGGKPD
VVQIVRNYNAYLRASDDL PKMFIESDPGFFSNAIVEGAKKFPNTEFVKVKGLHFSQED
APDEMGKYIKSFVERVLKNEQ**GSAGT**MYWEIEASEVMLSTRIGSGSFGTVYKGKWHG
DVAVKILKVVDPTPEQFQAFRNEVAVLRKTRHVNILLFMGYMTKDNLAIVTQWCEGSS
LYKHLHVQETKFQMFQLIDIARQTAQGMDYLHAKNIIHRDMKSNNIFLHEGLTVKIGD
FGLATVKSRWSGSQQVEQPTGSVLWMAPEVIRMQDNNPFSFQSDVYSYGIVLYELMTG
ELPYSHINNRDQIIFMVGRGYASPDLSKLYKNCPKAMKRLVADCVKKVKEERPLFPQI
LSSIELLQHSLPKINRSASEPSLHRAAHTEDINACTLTTSRPLPVFKMSKDGKKKKKK
SKTKCVIM (SEQ ID NO. 42)

Figure 20 cont.

Human KSR1 (hKSR1) sequences

>hKSR1-KD-wt CDS

CCCATCTCTCGCAAGGCCAGCCAGACCAGCGTGTACCTGCAGGAGTGGGACATCCCCT
 TCGAGCAGGTAGAGCTGGGCGAGCCCATCGGGCAGGGCCGCTGGGGCCGGGTGCACCG
 CGGCCGCTGGCATGGCGAGGTGGCCATTTCGCTGCTGGAGATGGACGGCCACAACCAG
 GACCACCTGAAGCTCTTCAAGAAAGAGGTGATGAACTACCGGCAGACGCGGCATGAGA
 ACGTGGTGCTCTTCATGGGGGCCTGCATGAACCCGCCCCACCTGGCCATTATCACCAG
 CTTCTGCAAGGGGCGGACGTTGCACTCGTTTGTGAGGGACCCCAAGACGTCTCTGGAC
 ATCAACAAGACGAGGCAAATCGCTCAGGAGATCATCAAGGGCATGGGATATCTTCATG
 CCAAGGGCATCGTACACAAAGATCTCAAATCTAAGAACGTCTTCTATGACAACGGCAA
 GGTGGTCATCACAGACTTCGGGCTGTTTGGGATCTCAGGCGTGGTCCGAGAGGGACGG
 CGTGAGAACCAGCTAAAGCTGTCCACGACTGGCTGTGCTATCTGGCCCCCTGAGATTG
 TACGCGAGATGACCCCCGGGAAGGACGAGGATCAGCTGCCATTCTCCAAAGCTGCTGA
 TGTCTATGCATTTGGGACTGTTTGGTATGAGCTGCAAGCAAGAGACTGGCCCTTGAAG
 AACCAGGCTGCAGAGGCATCCATCTGGCAGATTGGAAGCGGGGAAGGAATGAAGCGTG
 TCCTGACTTCTGTCAGCTTGGGGAAGGAAGTCAGTGAGATCCTGTCGGCCTGCTGGGC
 TTTCGACCTGCAGGAGAGACCCAGCTTCAGCCTGCTGATGGACATGCTGGAGAACTT
 CCAAGCTGAACCGGCGGCTCTCCACCCTGGACACTTCTGGAAGTCAGCTGAGTTGT
 AG (SEQ ID NO. 43)

>hKSR1-KD-wt

PISRKASQTSVYLQEWDPFEQVELGEPIGQGRWGRVHRGRWHGEVAIRLLEMDGHNQ
 DHLKLFKKEVMNYRQTRHENVVLFMGACMNPPHLAIITSFCKGRTLHSFVRDPKTSLD
 INKTRQIAQEIIKGMGYLHAKGIVHKDLKSKNVFYDNGKVITDFGLFGISGVVREGR
 RENQLKLSHDWLCYLAPEIVREMPGKDEDQLPFSKAADVYAFGTVWYELQARDWPLK
 NQAAEASIWQIGSGEGMKRVLTSSVSLGKEVSEILSACWAFDLQERPSFSLMLDMLEKL
 PKLNRRLSHPGHFWKSAEL (SEQ ID NO. 44)

>hKSR1-KD-C922Y CDS

ATGCCCATCTCTCGCAAGGCCAGCCAGACCAGCGTGTACCTGCAGGAGTGGGACATCC
 CCTTCGAGCAGGTAGAGCTGGGCGAGCCCATCGGGCAGGGCCGCTGGGGCCGGGTGCA
 CCGCGGCCGCTGGCATGGCGAGGTGGCCATTTCGCTGCTGGAGATGGACGGCCACAAC
 CAGGACCACCTGAAGCTCTTCAAGAAAGAGGTGATGAACTACCGGCAGACGCGGCATG
 AGAACGTGGTGCTCTTCATGGGGGCCTGCATGAACCCGCCCCACCTGGCCATTATCAC
 CAGCTTCTGCAAGGGGCGGACGTTGCACTCGTTTGTGAGGGACCCCAAGACGTCTCTG
 GACATCAACAAGACGAGGCAAATCGCTCAGGAGATCATCAAGGGCATGGGATATCTTC
 ATGCCAAGGGCATCGTACACAAAGATCTCAAATCTAAGAACGTCTTCTATGACAACGG
 CAAGGTGGTCATCACAGACTTCGGGCTGTTTGGGATCTCAGGCGTGGTCCGAGAGGGA
 CGGCGTGAGAACCAGCTAAAGCTGTCCACGACTGGCTGTGCTATCTGGCCCCCTGAGA
 TTGTACGCGAGATGACCCCCGGGAAGGACGAGGATCAGCTGCCATTCTCCAAAGCTGC
 TGATGTCTATGCATTTGGGACTGTTTGGTATGAGCTGCAAGCAAGAGACTGGCCCTTG
 AAGAACCAGGCTGCAGAGGCATCCATCTGGCAGATTGGAAGCGGGGAAGGAATGAAGC
 GTGTCCTGACTTCTGTCAGCTTGGGGAAGGAAGTCAGTGAGATCCTGTCGGCC**TAT**TG
 GGCTTTCGACCTGCAGGAGAGACCCAGCTTCAGCCTGCTGATGGACATGCTGGAGAAA
 CTTCCCAAGCTGAACCGGCGGCTCTCCACCCTGGACACTTCTGGAAGTCAGCTGAGT
 TGTAG (SEQ ID NO. 45)

Figure 21

>hKSR1-KD-C922Y
MPISRKASQTSVYLQEWDPFEQVELGEPIGQGRWGRVHRGRWHGEVAIRLLEMDGHN
QDHLKLFKKEVMNYRQTRHENVVLFMGACMNPPHLAIITSECKGRTLHSFVRDPKTSL
DINKTRQIAQEIIKGMGYLHAKGIVHKDLKSKNVFYDNGKVVITDFGLFGISGVVREG
RRENQLKLSHDWLCYLAPEIVREMPGKDEDQLPFSKAADVYAFGTVWYELQARDWPL
KNQAAEASIWQIGSGEGMKRVLTSVSLGKEVSEILSA~~Y~~WAFDLQERPSFSLLMDMLEK
LPKLNRRRLSHPGHFWKSAEL (SEQ ID NO. 46)

Figure 21 cont.

The human KSR1-KD-rlucII fusions are cloned between KpnI and PmeI in pCDNA3.1-zeo

>hKSR1-KD-wt-rlucII CDS

ATGCCCATCTCTCGCAAGGCCAGCCAGACCAGCGTGACCTGCAGGAGTGGGACATCC
CCTTCGAGCAGGTAGAGCTGGGCGAGCCCATCGGGCAGGGCCGCTGGGGCCGGGTGCA
CCGCGGCCGCTGGCATGGCGAGGTGGCCATTTCGCTGCTGGAGATGGACGGCCACAAC
CAGGACCACCTGAAGCTCTTCAAGAAAGAGGTGATGAACTACCGGCAGACGCGGCATG
AGAACGTGGTGCTCTTCATGGGGGCCTGCATGAACCCGCCCCACCTGGCCATTATCAC
CAGCTTCTGCAAGGGGCGGACGTTGCACTCGTTTGTGAGGGACCCCAAGACGTCTCTG
GACATCAACAAGACGAGGCAAATCGCTCAGGAGATCATCAAGGGCATGGGATATCTTC
ATGCCAAGGGCATCGTACACAAAGATCTCAAATCTAAGAACGTCTTCTATGACAACGG
CAAGGTGGTCATCACAGACTTCGGGCTGTTTGGGATCTCAGGCGTGGTCCGAGAGGGA
CGGCGTGAGAACCAGCTAAAGCTGTCCACGACTGGCTGTGCTATCTGGCCCCTGAGA
TTGTACGCGAGATGACCCCCGGGAAGGACGAGGATCAGCTGCCATTCTCCAAAGCTGC
TGATGTCTATGCATTTGGGACTGTTTGGTATGAGCTGCAAGCAAGAGACTGGCCCTTG
AAGAACCAGGCTGCAGAGGCATCCATCTGGCAGATTGGAAGCGGGGAAGGAATGAAGC
GTGTCCTGACTTCTGTCAGCTTGGGGAAGGAAGTCAGTGAGATCCTGTCGGCCTGCTG
GGCTTTCGACCTGCAGGAGAGACCCAGCTTCAGCCTGCTGATGGACATGCTGGAGAAA
CTTCCCAAGCTGAACCGGCGGCTCTCCACCCCTGGACACTTCTGGAAGTCAGCTGAGT
TGTCCTAGAGGAGGGGGGATGACCAGCAAGGTGTACGACCCCGAGCAGAGGAAGAGGAT
GATCACCGGCCCCCAGTGGTGGGCCAGGTGCAAGCAGATGAACGTGCTGGACAGCTTC
ATCAACTACTACGACAGCGAGAAGCACGCCGAGAACGCCGTGATCTTCCTGCACGGCA
ACGCCACTAGCAGCTACCTGTGGAGGCACGTGGTGCCCCACATCGAGCCCGTGGCCAG
GTGCATCATCCCCGATCTGATCGGCATGGGCAAGAGCGGCAAGAGCGGCAACGGCAGC
TACAGGCTGCTGGACCACTACAAGTACCTGACCGCCTGGTTCGAGCTCCTGAACCTGC
CCAAGAAGATCATCTTCGTGGGCCACGACTGGGGCGCCGCACTGGCCTTCCACTACAG
CTACGAGCACCAGGACAAGATCAAGGCCATCGTGACGCCGAGAGCGTGGTTGACGTG
ATCGAGAGCTGGGACGAGTGGCCAGACATCGAGGAGGACATCGCCCTGATCAAGAGCG
AGGAGGGCGAGAAGATGGTGCTGGAGAACAACCTTCTTCGTGGAGACCGTTCTGCCCAG
CAAGATCATGAGAAAGCTGGAGCCCGAGGAGTTCGCCGCCTACCTGGAGCCCTTCAAG
GAGAAGGGCGAGGTGAGAAGACCCACCCTGAGCTGGCCCAGAGAGATCCCCCTGGTGA
AGGGCGGCAAGCCCCGACGTGGTGCAGATCGTGAGAACTACAACGCCTACCTGAGAGC
CAGCGACGACCTGCCCAAGATGTTTCATCGAGAGCGACCCCGGCTTCTTCAGCAACGCC
ATCGTGGAGGGCGCCAAGAAGTTCCCAACACCGAGTTCGTGAAGGTGAAGGGCCTGC
ACTTCAGCCAGGAGGACGCCCCCGACGAGATGGGCAAGTACATCAAGAGCTTCGTGGA
GAGAGTGCTGAAGAACGAGCAGTAG (SEQ ID NO. 47)

Figure 22

>hKSR1-KD-wt-rlucII

MPISRKASQTSVYLQEWDIPFEQVELGEPIGQGRWGRVHRGRWHGEVAIRLLEMDGHN
QDHLKLFKKEVMNYRQTRHENVVLEFMGACMNPPHLAIITSFCKGRTLHSFVRDPKTSL
DINKTRQIAQEIIKGMGYLHAKGIVHKDLKSKNVFYDNGKVVITDFGLFGISGVVREG
RRENQLKLSHDWLCYLAPEIVREMPGKDEDQLPFSKAADVYAFGTVWYELQARDWPL
KNQAAEASIWQIGSGEGMKRVLTSLGKEVSEILSACWAFDLQERPSFSLLMDMLEK
LPKLNRRLSHPGHFWKSAEL**SRGGG**MTSKVYDPEQRKRMITGPQWWARCKQMNVLDSF
INYYDSEKHAENAVIFLHGNATSSYLWRHVPHIEPVARCIIPDLIGMGKSGKSGNGS
YRLLDHYKYLTAWFELNLPKKII FVGHDWGAALAFHYSYEHQDKIKAIVHAESVVDV
IESWDEWPDIEEDIALIKSEEGEKMLENNFFVETVLPSKIMRKLEPEEFAAYLEPFK
EKGEVRRPTLSWPREIPLVKGGKPDVVQIVRNYNAYLRASDDLPKMFIESDPGFFSNA
IVEGAKKFPNTEFVKVKGLHFSQEDAPDEMCKYIKSFVERVLKNEQ- (SEQ ID NO.
48)

>hKSR1-KD-C922Y-rlucII CDS

ATGCCCATCTCTCGCAAGGCCAGCCAGACCAGCGTGTACCTGCAGGAGTGGGACATCC
CCTTCGAGCAGGTAGAGCTGGGCGAGCCCATCGGGCAGGGCCGCTGGGGCCGGGTGCA
CCGCGGCCGCTGGCATGGCGAGGTGGCCATTTCGCCTGCTGGAGATGGACGGCCACAAC
CAGGACCACCTGAAGCTCTTCAAGAAAGAGGTGATGAACTACCGGCAGACGCGGCATG
AGAACGTGGTGCTCTTCATGGGGGCGCTGCATGAACCCGCCCCACCTGGCCATTATCAC
CAGCTTCTGCAAGGGGCGGACGTTGCACTCGTTTGTGAGGGACCCCAAGACGTCTCTG
GACATCAACAAGACGAGGCAAATCGCTCAGGAGATCATCAAGGGCATGGGATATCTTC
ATGCCAAGGGCATCGTACACAAAGATCTCAAATCTAAGAACGTCTTCTATGACAACGG
CAAGGTGGTCATCACAGACTTCGGGCTGTTTGGGATCTCAGGCGTGGTCCGAGAGGGA
CGGCGTGAGAACCAGCTAAAGCTGTCCACGACTGGCTGTGCTATCTGGCCCCTGAGA
TTGTACGCGAGATGACCCCCGGGAAGGACGAGGATCAGCTGCCATTCTCCAAAGCTGC
TGATGTCTATGCATTTGGGACTGTTTGGTATGAGCTGCAAGCAAGAGACTGGCCCTTG
AAGAACCAGGCTGCAGAGGCATCCATCTGGCAGATTGGAAGCGGGGAAGGAATGAAGC
GTGTCCTGACTTCTGTCAGCTTGGGGAAGGAAGTCAGTGAGATCCTGTCGGCC**TATTG**
GGCTTTTCGACCTGCAGGAGAGACCCAGCTTCAGCCTGCTGATGGACATGCTGGAGAAA
CTTCCCAAGCTGAACCGGCGGCTCTCCACCCCTGGACACTTCTGGAAGTCAGCTGAGT
TG**TCTAGAGGAGGGGGG**ATGACCAGCAAGGTGTACGACCCCGAGCAGAGGAAGAGGAT
GATCACCGGCCCCCAGTGGTGGGCCAGGTGCAAGCAGATGAACGTGCTGGACAGCTTC
ATCAACTACTACGACAGCGAGAAGCACGCCGAGAACGCCGTGATCTTCCTGCACGGCA
ACGCCACTAGCAGCTACCTGTGGAGGCACGTGGTGCCCCACATCGAGCCCGTGGCCAG
GTGCATCATCCCCGATCTGATCGGCATGGGCAAGAGCGGCAAGAGCGGCAACGGCAGC
TACAGGCTGCTGGACCACTACAAGTACCTGACCGCCTGGTTCGAGCTCCTGAACCTGC
CCAAGAAGATCATCTTCGTGGGCCACGACTGGGGCGCCGCACTGGCCTTCCACTACAG
CTACGAGCACCAGGACAAGATCAAGGCCATCGTGCACGCCGAGAGCGTGGTTGACGTG
ATCGAGAGCTGGGACGAGTGGCCAGACATCGAGGAGGACATCGCCCTGATCAAGAGCG
AGGAGGGCGAGAAGATGGTGCTGGAGAACAACCTTCTTCGTGGAGACCGTTCTGCCCAG
CAAGATCATGAGAAAGCTGGAGCCCGAGGAGTTCGCCGCCTACCTGGAGCCCTTCAAG
GAGAAGGGCGAGGTGAGAAGACCCACCCTGAGCTGGCCCAGAGAGATCCCCCTGGTGA
AGGGCGGCAAGCCCGACGTGGTGCAGATCGTGAGAACTACAACGCCTACCTGAGAGC
CAGCGACGACCTGCCCAAGATGTTTCATCGAGAGCGACCCCGGCTTCTTCAGCAACGCC
ATCGTGAGAGGGCGCCAAGAAGTTCCTCCCAACACCGAGTTCGTGAAGGTGAAGGGCCTGC
ACTTCAGCCAGGAGGACGCCCCGACGAGATGGGCAAGTACATCAAGAGCTTCGTGGA
GAGAGTGCTGAAGAACGAGCAGTAG (SEQ ID NO. 49)

Figure 22 cont.

>hKSR1-KD-C922Y-rlucII
MPISRKASQTSVYLQEWDPFEQVELGEPIGQGRWGRVHRGRWHGEVAIRLLEMDGHN
QDHLKLFKKEVMNYRQTRHENVVLEFMGACMNPPHLAIITSFCKGRTLHSFVRDPKTSL
DINKTRQIAQEIIKGMGYLHAKGIVHKDLKSKNVFYDNGKVVITDFGLFGISGVVREG
RRENQLKLSHDWLCYLAPEIVREMPGKDEDQLPFASKAADVYAFGTVWYELQARDWPL
KNQAAEASIWQIGSGEGMKRVLTSLGKEVSEILSAWAFDLQERPSFSLLMDMLEK
LPKLNRRLSHPGHFWKSAEL**SRGGG**MTSKVYDPEQRKRMITGPQWWARCKQMNVLDSF
INYDSEKHAENAVIFLHGNATSSYLWRHVPHIEPVARCIIPDLIGMGKSGKSGNGS
YRLLDHYKYLTAWFELLNLPKKIIFVGHDWGAALAFHYSYEHQDKIKAIVHAESVVDV
IESWDEWPDIEEDIALIKSEEKGMVLENNFFVETVLPSKIMRKLEPEEFAAYLEPFK
EKGEVRRPTLSWPREIPLVKGGKPDVVQIVRNYNAYLRASDDLPMFIESDPGFFSNA
IVEGAKKFPNTEFVKVKGLHFSQEDAPDEMGKYIKSFVERVLKNEQ (SEQ ID NO.
50)

Figure 22 cont.

Human MEK1 (hMEK1)

>hMEK1 CDS

ATGCCCAAGAAGAAGCCGACGCCCATCCAGCTGAACCCGGCCCCCGACGGCTCTGCAG
 TTAACGGGACCAGCTCTGCGGAGACCAACTTGGAGGCCTTGCAGAAGAAGCTGGAGGA
 GCTAGAGCTTGATGAGCAGCAGCGAAAGCGCCTTGAGGCCTTTCTTACCCAGAAGCAG
 AAGGTGGGAGAACTGAAGGATGACGACTTTGAGAAGATCAGTGAGCTGGGGGCTGGCA
 ATGGCGGTGTGGTGTTC AAGGTCTCCACAAGCCTTCTGGCCTGGTCATGGCCAGAAA
 GCTAATTCATCTGGAGATCAAACCCGCAATCCGGAACCAGATCATAAGGGAGCTGCAG
 GTTCTGCATGAGTGCAACTCTCCGTACATCGTGGGCTTCTATGGTGCGTTCTACAGCG
 ATGGCGAGATCAGTATCTGCATGGAGCACATGGATGGAGGTTCTCTGGATCAAGTCCT
 GAAGAAAGCTGGAAGAATTCCTGAACAAATTTTAGGAAAAGTTAGCATTGCTGTAATA
 AAAGGCCTGACATATCTGAGGGGAGAAGCACAAAGATCATGCACAGAGATGTCAAGCCCT
 CCAACATCCTAGTCAACTCCCGTGGGGAGATCAAGCTCTGTGACTTTGGGGTCAGCGG
 GCAGCTCATCGACTCCATGGCCAACTCCTTCGTGGGCACAAGGTCCTACATGTCGCCA
 GAAAGACTCCAGGGGACTCATTACTCTGTGCAGTCAGACATCTGGAGCATGGGACTGT
 CTCTGGTAGAGATGGCGGTTGGGAGGTATCCCATCCCTCCTCCAGATGCCAAGGAGCT
 GGAGCTGATGTTTGGGTGCCAGGTGGAAGGAGATGCGGCTGAGACCCACCCAGGCCA
 AGGACCCCCGGGAGGCCCCCTTAGCTCATAACGGAATGGACAGCCGACCTCCCATGGCAA
 TTTTTGAGTTGTTGGATTACATAGTCAACGAGCCTCCTCCAAAACCTGCCCAGTGGAGT
 GTTCAGTCTGGAATTTCAAGATTTTGTGAATAAATGCTTAATAAAAAACCCCGCAGAG
 AGAGCAGATTTGAAGCAACTCATGGTTCATGCTTTTATCAAGAGATCTGATGCTGAGG
 AAGTGGATTTTGCAGGTTGGCTCTGCTCCACCATCGGCCTTAACCAGCCCAGCACACC
 AACCCATGCTGCTGGCGTCTAA (SEQ ID NO. 51)

>hMEK1

MPKKKPTPIQLNPAPDGS AVNGTSSAETNLEALQKKLEEELELDEQQRKRLEAFLTQKQ
 KVGELKDDDFEKISELGAGNGGVVFKVSHKPSGLVMARKLIHLEIKPAIRNQIIRELQ
 VLHECNSPYIVGFYGA FYSDGEISICMEHMDGGS LDQVLKKAGRIPEQILGKVSIAVI
 KGLTYLREKHKIMHRDVKPSN ILVNSRGEIKLCDFGVSGQLIDSMANSFVGTRSYMSP
 ERLQGTHYSVQSDIWSMGLSLVEMAVGRYP I PPPDAKELELMFGCQVEGDAAETPPRP
 RTPGRPLSSYGMDSRPPMAIFELLDYIVNEPPPKLPSGVFSLEFQDFVNKCLIKNP AE
 RADLKQLMVHAFIKRSDAE EVDFAGWLCSTIGLNQ PSTP THAAGV (SEQ ID NO.
 52)

Figure 23

The human GFP10-MEK1 full length fusion is cloned between NheI and XbaI in pCDNA3.1-zeo

>GFP10-hMEK1 CDS

CACCATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTCGAG
CTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATG
CCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCCGTGCC
CTGGCCCCACCCTCGTGACCACCCTGAGCTACGGCGTGCAGTGCTTCAGCCGCTACCCC
GACCACATGAAGCAGCAGACTTCTTCAAGTCCGCCATGCCCCGAAGGCTACGTCCAGG
AGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTT
CGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGAC
GGCAACATCCTGGGGCACAAGCTGGAGTACAACCTACAACCCCCACAACGTCTATATCA
TGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGA
GGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGC
CCCGTGCTGCTGCCCCGACAACCACTACCTGTTACCCAGTCCGCCCTGAGCAAAGACC
CCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCAC
TCTCGGCATGGACGAGCTGTACAAG**GGATCCGCGGTAC**CCCCAAGAAGAAGCCGACG
CCCATCCAGCTGAACCCGGCCCCCGACGGCTCTGCAGTTAACGGGACCAGCTCTGCGG
AGACCAACTTGGAGGCCTTGCAGAAGAAGCTGGAGGAGCTAGAGCTTGATGAGCAGCA
GCGAAAGCGCCTTGAGGCCTTTCTTACCCAGAAGCAGAAGGTGGGAGAACTGAAGGAT
GACGACTTTGAGAAGATCAGTGAGCTGGGGGCTGGCAATGGCGGTGTGGTGTTCAGG
TCTCCACAAGCCTTCTGGCCTGGTCATGGCCAGAAAGCTAATTCATCTGGAGATCAA
ACCCGCAATCCGGAACCAGATCATAAGGGAGCTGCAGGTTCTGCATGAGTGCAACTCT
CCGTACATCGTGGGCTTCTATGGTGCGTTCTACAGCGATGGCGAGATCAGTATCTGCA
TGGAGCACATGGATGGAGGTTCTCTGGATCAAGTCCTGAAGAAAGCTGGAAGAATTCC
TGAACAAATTTTAGGAAAAGTTAGCATTGCTGTAATAAAAGGCCTGACATATCTGAGG
GAGAAGCACAAGATCATGCACAGAGATGTCAAGCCCTCCAACATCCTAGTCAACTCCC
GTGGGGAGATCAAGCTCTGTGACTTTGGGGTCAGCGGGCAGCTCATCGACTCCATGGC
CAACTCCTTCGTGGGCACAAGGTCCTACATGTCGCCAGAAAGACTCCAGGGGACTCAT
TACTCTGTGCAGTCAGACATCTGGAGCATGGGACTGTCTCTGGTAGAGATGGCGGTTG
GGAGGTATCCCATCCCTCCTCCAGATGCCAAGGAGCTGGAGCTGATGTTTGGGTGCCA
GGTGGAAGGAGATGCGGCTGAGACCCCACCCAGGCCAAGGACCCCCGGGAGGCCCTT
AGCTCATACGGAATGGACAGCCGACCTCCCATGGCAATTTTTGAGTTGTTGGATTACA
TAGTCAACGAGCCTCCTCCAAAACCTGCCAGTGGAGTGTTCAAGTCTGGAATTTCAAGA
TTTTGTGAATAAATGCTTAATAAAAAACCCCGCAGAGAGAGCAGATTTGAAGCAACTC
ATGGTTCATGCTTTTATCAAGAGATCTGATGCTGAGGAAGTGGATTTTGCAGGTTGGC
TCTGCTCCACCATCGGCCTTAACCAGCCCAGCACACCAACCCATGCTGCTGGCGTCTA
A (SEQ ID NO. 53)

Figure 24

>GFP10-hMEK1
MVSKEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTTLKFICTTGKLPVPW
PTLVTTLSYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFE
GDTLVNRIELKGIDFKEDGNILGHKLEYNYPHNVYIMADKQKNGIKVNFKIRHNIED
GSVQLADHYQQNTPIGDGPVLLPDNHYLFTQSALSKDPNEKRDHMLLEFVTAAGITL
GMDELYK**GSAGT**PKKKPTPIQLNPAPDGSVNGTSSAETNLEALQKKLEEELELDEQQR
KRLEAFLTQKQKVGELKDDDFEKISELGAGNGGVVFKVSHKPSGLVMARKLIHLEIKP
AIRNQIIRELQVLHECNSPYIVGFYGAFYSDGEISICMEHMDGGSLDQVLKKAGRIPE
QILGKVSIAVIKGLTYLREKHKIMHRDVKPSNILVNSRGEIKLCDFGVSGQLIDSMAN
SFVGTRSYMSPERLQGTHYSVQSDIWSMGLSLVEMAVGRYPPIPPDAKELELMFGCQV
EGDAAETPPRPRTTPGRPLSSYGMDSRPPMAIFELLDYIVNEPPPKLPSGVFSLEFQDF
VНКCLIKNPAERADLKQLMVHAFIKRSDAEEVDFAGWLCSTIGLNQPSTPTHAAAGV-
(SEQ ID NO. 54)

Figure 24 cont.

CLONING OLIGONUCLEOTIDES:**Human BRAF**

>OL5_hBRAF_KD_+start_F

GGGGTACCATGGATGATTGGGAGATTCCTGATGGGC (SEQ ID NO. 55)

>OL3_hBRAF_KD_+CAAX_+stop_R

GCTCTAGATTACATAATTACACACTTTGTCTTTGACTTCTTTTTCTTCTTTTACCAT
CTTTGCTCATCTTGTGGACAGGAAACGCACCATA (SEQ ID NO. 56)**Human CRAF**

>OL5_hCRAF_KD_+start_F

GGGGTACCATGTATTATTGGGAAATAGAAGCC (SEQ ID NO. 57)

>OL3_hCRAF_KD_+CAAX_+stop_R

GCTCTAGATTACATAATTACACACTTTGTCTTTGACTTCTTTTTCTTCTTTTACCAT
CTTTGCTCATCTTGAAGACAGGCAGCCTCGGGGA (SEQ ID NO. 58)**Human KSR1**

>OL5_hKSR1_KpnI_cloning_BRET

GGGGTACCCCCATCTCTCGCAAGGCCAGCCAG (SEQ ID NO. 59)

>OL3_hKSR1_XbaI_cloning_BRET

GCTCTAGACTACAACCTCAGCTGACTTCCAGAAG (SEQ ID NO. 60)**Human MEK**

>OL5_hMEK1_KpnI_cloning_BRET

GGGGTACCCCCAAGAAGAAGCCGACGCCCATC (SEQ ID NO. 61)

>OL3_hMEK1_XbaI_cloning_BRET

GCTCTAGATTAGACGCCAGCAGCATGGGTTGG (SEQ ID NO. 62)

Figure 25

SEQUENCING OLIGONUCLEOTIDES:

>OL5_hBRAF_seq1_F
GGGTTTCCGCTGTCAAACATGTGG (SEQ ID NO. 63)

>OL5_hBRAF_seq2_F
TATTGTTACCCAGTGGTGTGAGGG (SEQ ID NO. 64)

>OL5_hCRAF_seq1_F
ACACCTAATGTCCACATGGTCAGC (SEQ ID NO. 65)

>OL5_hCRAF_seq2_F
TTTCCAGTCGGATGTCTACTCCTA (SEQ ID NO. 66)

Figure 26

MUTAGENESIS OLIGONUCLEOTIDES:

The following primer pair was used to generate the side-to-side dimer interface mutant R481H in BRAF

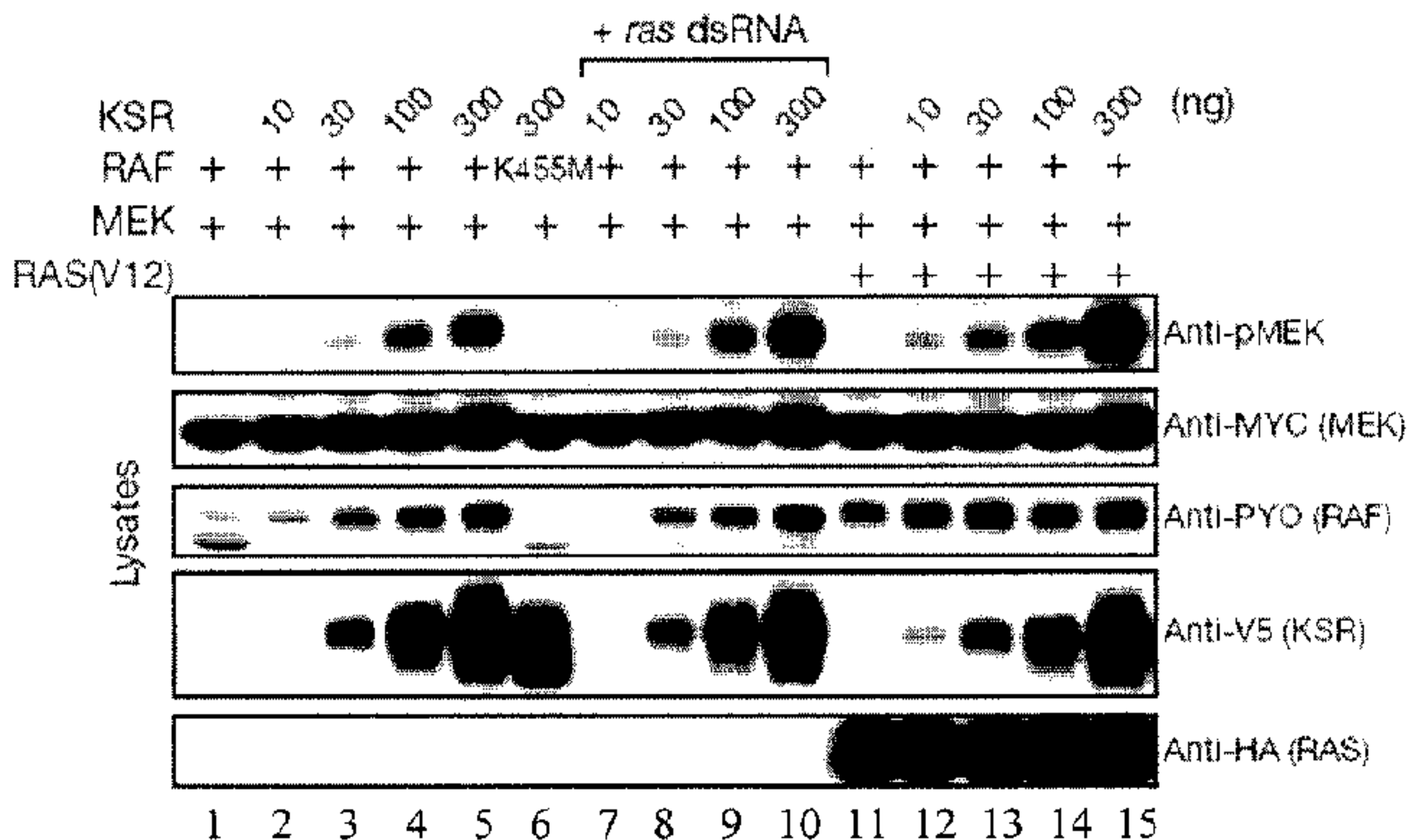
>OL5_hBRAF_R481H_F
AATGAAGTAGGAGTACTCAGGAAAACAC**CAT**CATGTGAATATCCTACTCTTCATGGGC
(SEQ ID NO. 67)
>OL3_hBRAF_R481H_R
GCCCATGAAGAGTAGGATATTCACATG**ATGT**GTTTTCTGAGTACTCCTACTTCATT
(SEQ ID NO. 68)

The following primer pair was used to introduce the C922Y hMEK1 interaction mutant in hKSR1

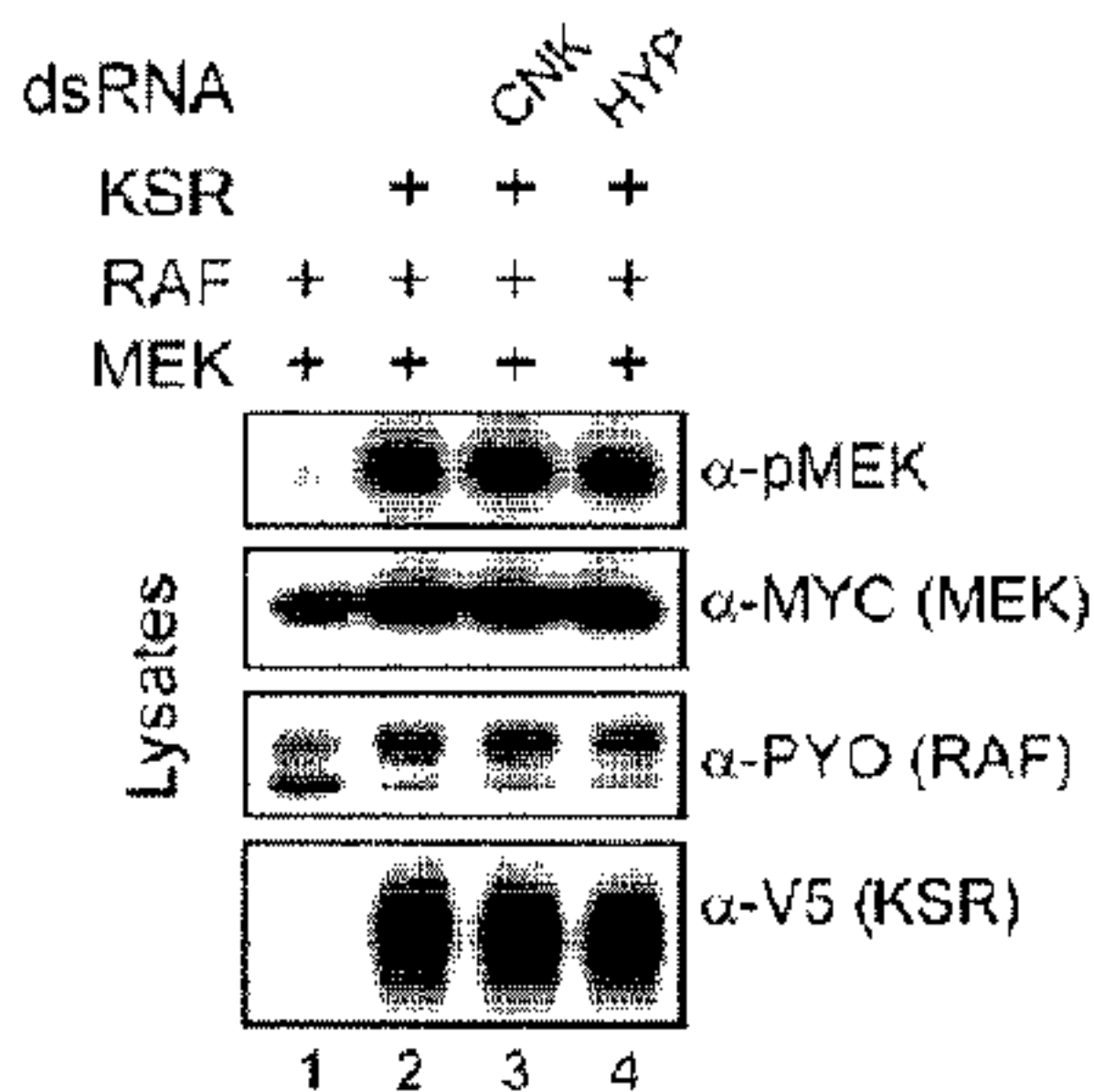
>OL5_hKSR1_C922Y_F
GAAGTCAGTGAGATCCTGTCGGCC**TATT**GGGCTTTTCGACCTGCAGGAGAGA (SEQ
ID NO. 69)
>OL3_hKSR1_C922Y_R
TCTCTCCTGCAGGTCGAAAGCCCA**ATAG**GCCGACAGGATCTCACTGACTTC (SEQ
ID NO. 70)

Figure 27

A



B



C

