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Supplemental Information

Distinct Binding Preferences between Ras and Raf Family Members and the Impact on Oncogenic Ras Signaling

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SUPPLEMENTAL FIGURES

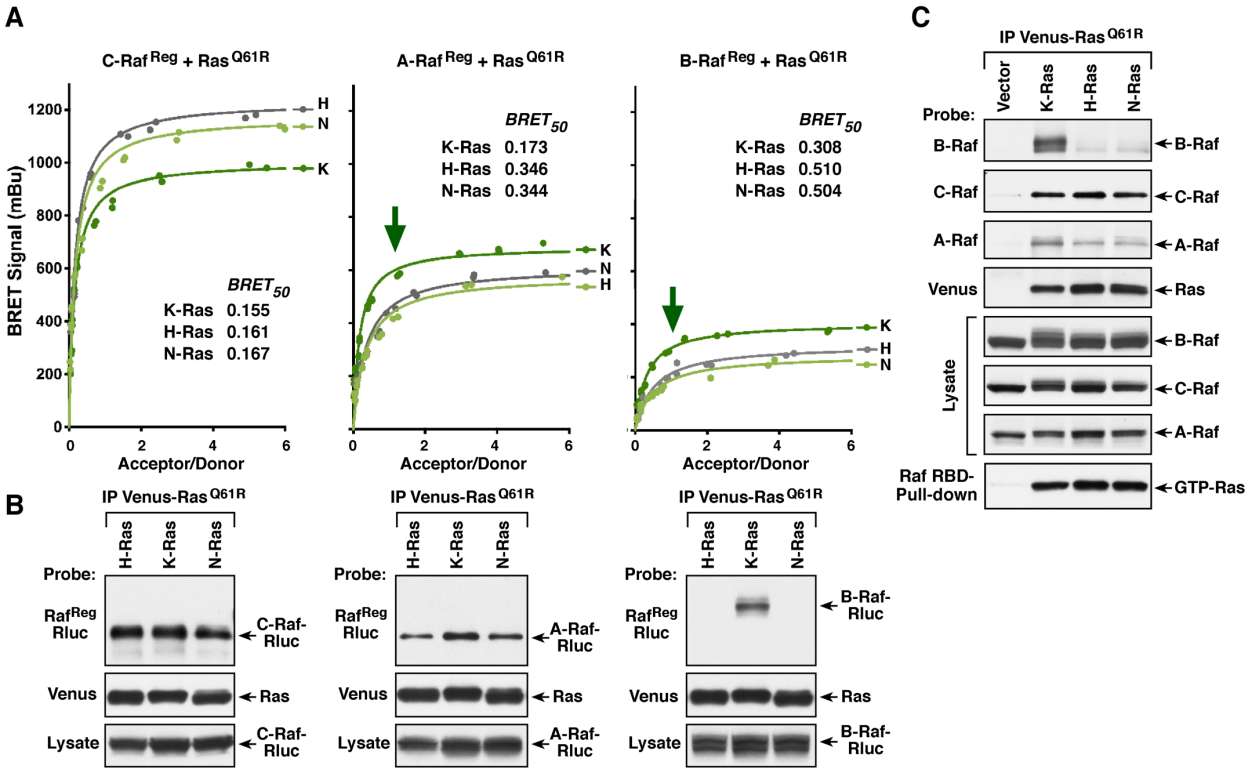

Figure S1, related to Figure 2. BRET Analysis of Binding Interactions Between the Raf Family Members and Mutant Ras Proteins

(A) BRET saturation curves were performed in 293FT cells to examine the interaction of C-Raf (left), A-Raf (middle), or B-Raf (right) regulatory domain proteins (Reg) with Q61R mutants of H-Ras, K-Ras, and N-Ras. The Rafs functioned as the energy donor tagged at the C-terminus with the Rluc8 enzyme and the Ras proteins served as the energy acceptor tagged at the N-terminus with the Venus fluorophore. $BRET_{50}$ values are also listed. (B) The BRET-tagged Ras and Raf proteins analyzed in (A) were examined in co-immunoprecipitation assays. (C) Venus-Ras^{Q61R} complexes were immunoprecipitated from 293FT cell lysates and probed for the presence of endogenous B-Raf, C-Raf, or A-Raf and Venus-Ras by immunoblot analysis. Venus-Ras^{Q61R} proteins were also examined for GTP-loading in Raf RBD pull-down assays, and lysates were examined for B-Raf, C-Raf, and A-Raf levels. These findings demonstrate the distinct binding preferences of the Raf and Ras family members.

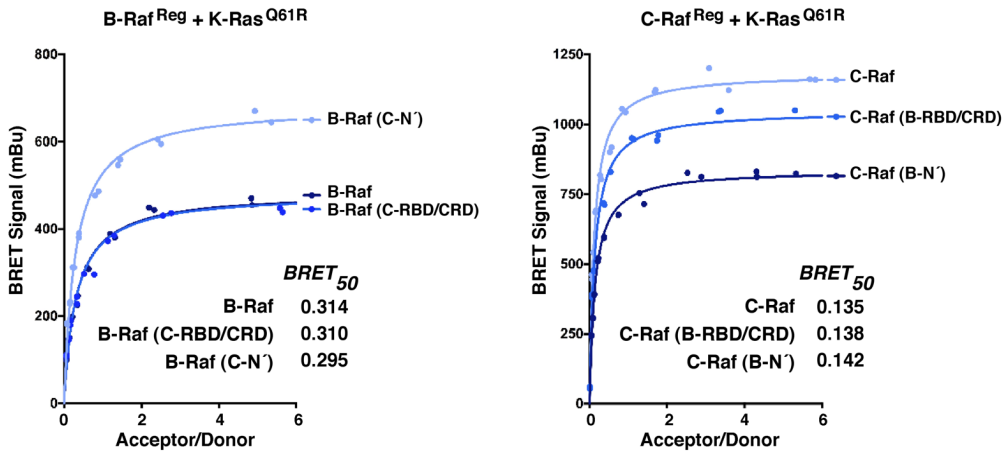

Figure S2, related to Figure 3. Effect of Raf N´-Segment on the Raf/K-Ras^{Q61R} Interaction

Raf proteins were generated in which regions of the B-Raf and C-Raf regulatory domains were exchanged. Shown are BRET saturation curves examining the interaction of WT- or domain-exchanged Raf^{Reg}-Rluc proteins with Venus-K-Ras^{Q61R}. $BRET_{50}$ values are also listed. The presence of the B-Raf N´-segment reduced the $BRET_{max}$ of the Raf/K-Ras^{Q61R} interaction but had no significant effect on the $BRET_{50}$ values.

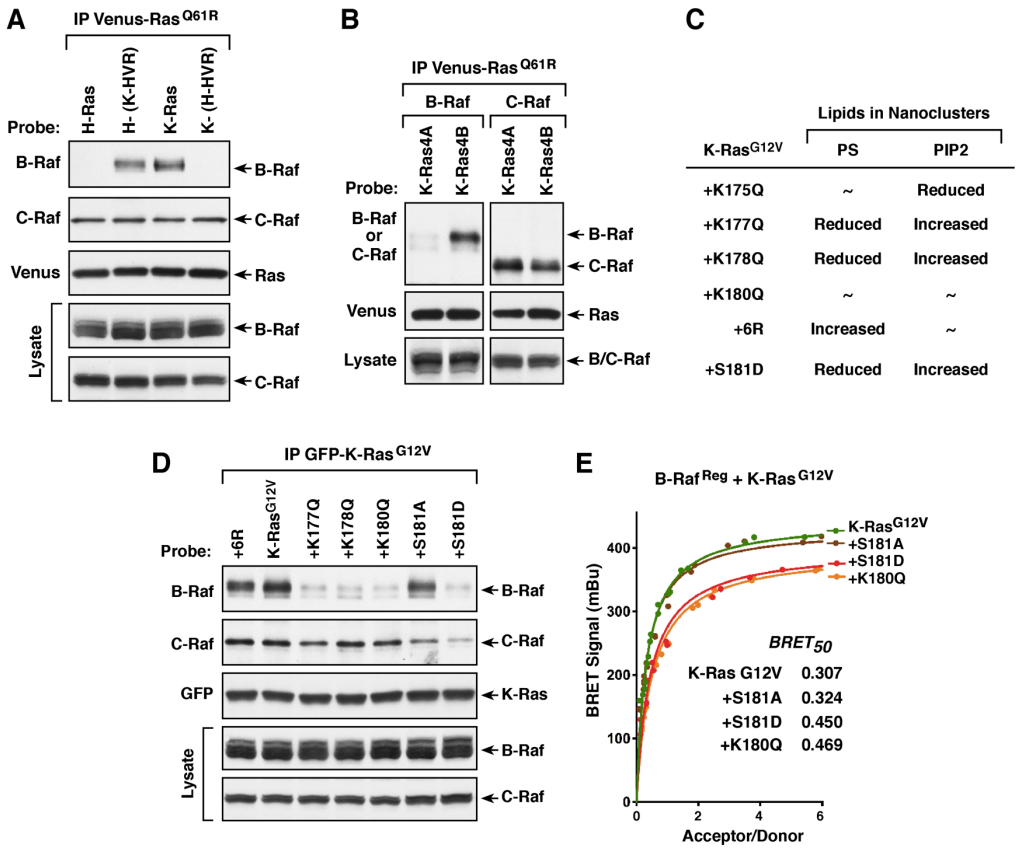

Figure S3, related to Figure 4. Analysis of B-Raf/Ras Binding Requirements

(A) WT or HVR-exchanged Venus-Ras^{Q61R} proteins were immunoprecipitated from 293FT cell lysates and examined for the presence of endogenous B-Raf or C-Raf and Venus-Ras. Endogenous B-Raf selectively interacts with mutant Ras proteins that contain the K-Ras HVR, whereas endogenous C-Raf interacts with all Ras proteins regardless of the HVR origin. (B) Venus-tagged K-Ras4A^{Q61R} or K- Ras4B^{Q61R} proteins were immunoprecipitated from 293FT cell lysates and examined for the presence of endogenous B-Raf or C-Raf and Venus-Ras. Endogenous C-Raf, but not B-Raf, efficiently co-immunoprecipitates with the K-Ras4A splice variant, which lacks the PBR sequences. (C) Listed are the reported effects of the PBR mutations on the lipid composition of K-Ras^{G12V} membrane nanoclusters. (D) GFP-tagged K-Ras^{G12V} complexes were immunoprecipitated from cell lysates and examined for the presence of endogenous B-Raf or C-Raf and GFP-K-Ras^{G12V}. Binding of endogenous B-Raf was lower to the PBR mutants that have a reduced positive charge. (E) BRET saturation curves are shown examining the interaction of Raf^{Reg}-Rluc with the indicated Venus-Ras^{G12V} proteins. $BRET_{50}$ values are also listed.

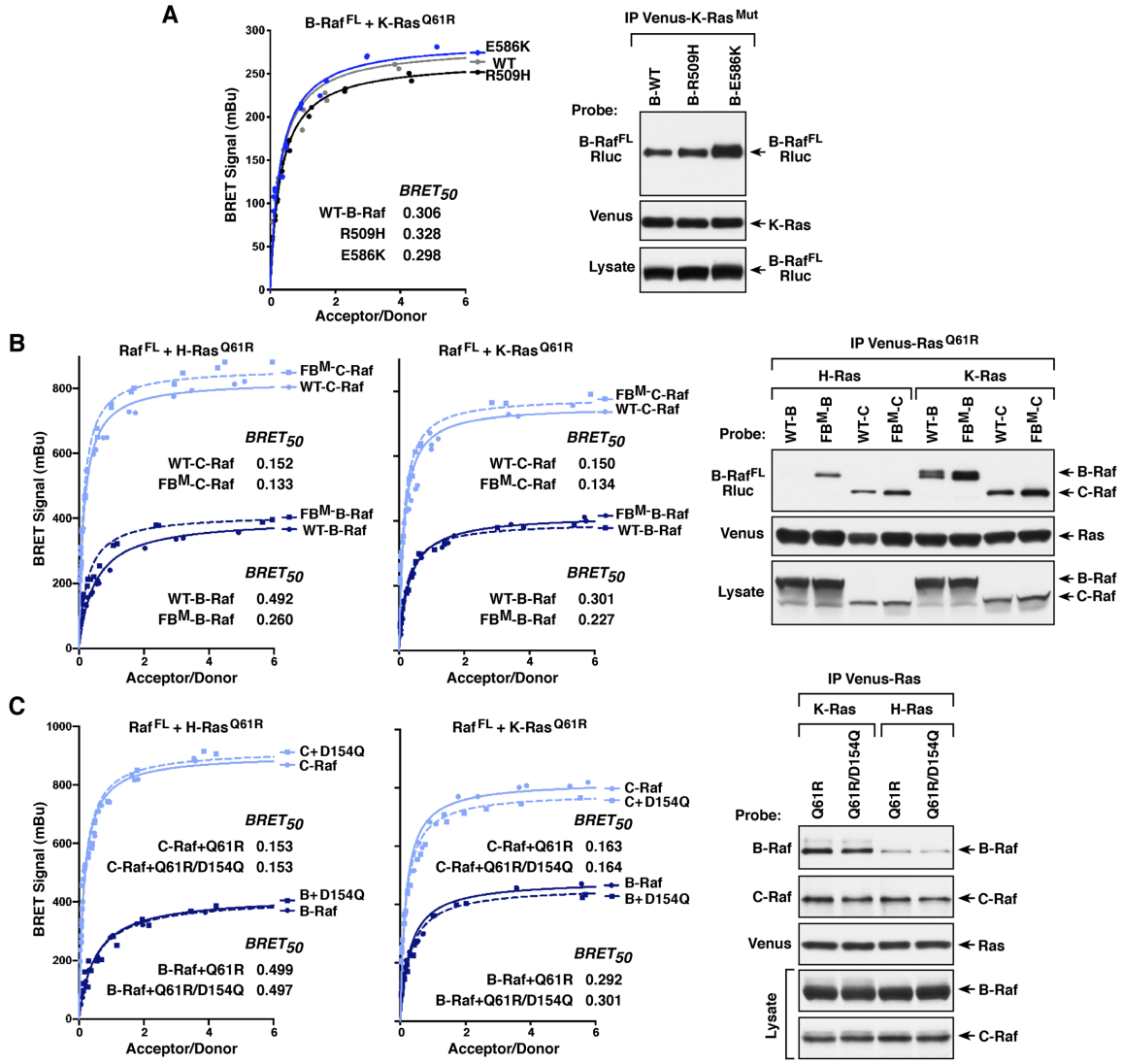

Figure S4, related to Figure 5. Effect of B-Raf/C-Raf Dimerization on Ras/Raf Binding

(A) BRET (left) and co-immunoprecipitation assays (right) were performed in 293FT cells examining the interaction of WT, R509H (dimer-defective), or E586K (dimer-enhanced) B-Raf^{FL}-Rluc proteins with Venus-K-Ras^{Q61R}. (B) BRET (left) and coimmunoprecipitation assays (right) were performed examining the ability of WT Raf^{FL}-Rluc or Raf proteins containing mutations in the ERK-induced feedback phosphorylation sites (FB^{M}-B-Raf^{FL}-Rluc or FB^{M}-C-Raf^{FL}-Rluc) to interact with Venus-tagged H-Ras^{Q61R} or K-Ras^{Q61R}. (C) BRET (left) and co-immunoprecipitation assays (right) were performed examining the interaction of B-Raf^{FL}-Rluc or C-Raf^{FL}-Rluc with Venus-tagged H-Ras^{Q61R}, K-Ras^{Q61R}, H-Ras$^{Q61R/D154Q}$ or K-Ras$^{Q61R/D154Q}$. Events that alter B-Raf/C-Raf dimer formation, but not Ras dimerization, impact the Ras/Raf interaction.

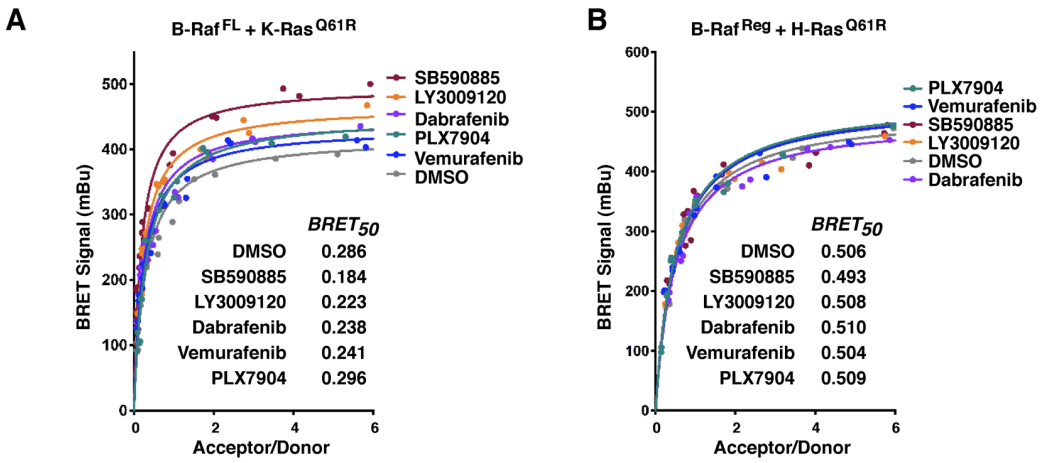

Figure S5, related to Figure 5. Effect of Raf Inhibitor Treatment on Ras/Raf Binding Interactions in Live Cells

(A) 293FT cells expressing full-length B-Raf^{FL}-Rluc and Venus-K-Ras^{Q61R} were treated for 1 hr with DMSO or the indicated ATP-competitive Raf inhibitors at 1 μM concentration before BRET measurements were obtained. BRET saturation curves and $BRET_{50}$ values are shown depicting the effect of Raf inhibitor treatment on the B-Raf/K-Ras interaction. (B) 293FT cells expressing the B-Raf regulatory domain B-Raf^{Reg}-Rluc protein and Venus-H-Ras^{Q61R} were treated as in (A) prior to obtaining the BRET measurements. The BRET results indicate that the effect of the Raf inhibitors on the B-Raf/H-Ras^{Q61R} interaction requires binding of the compounds to the B-Raf catalytic domain.

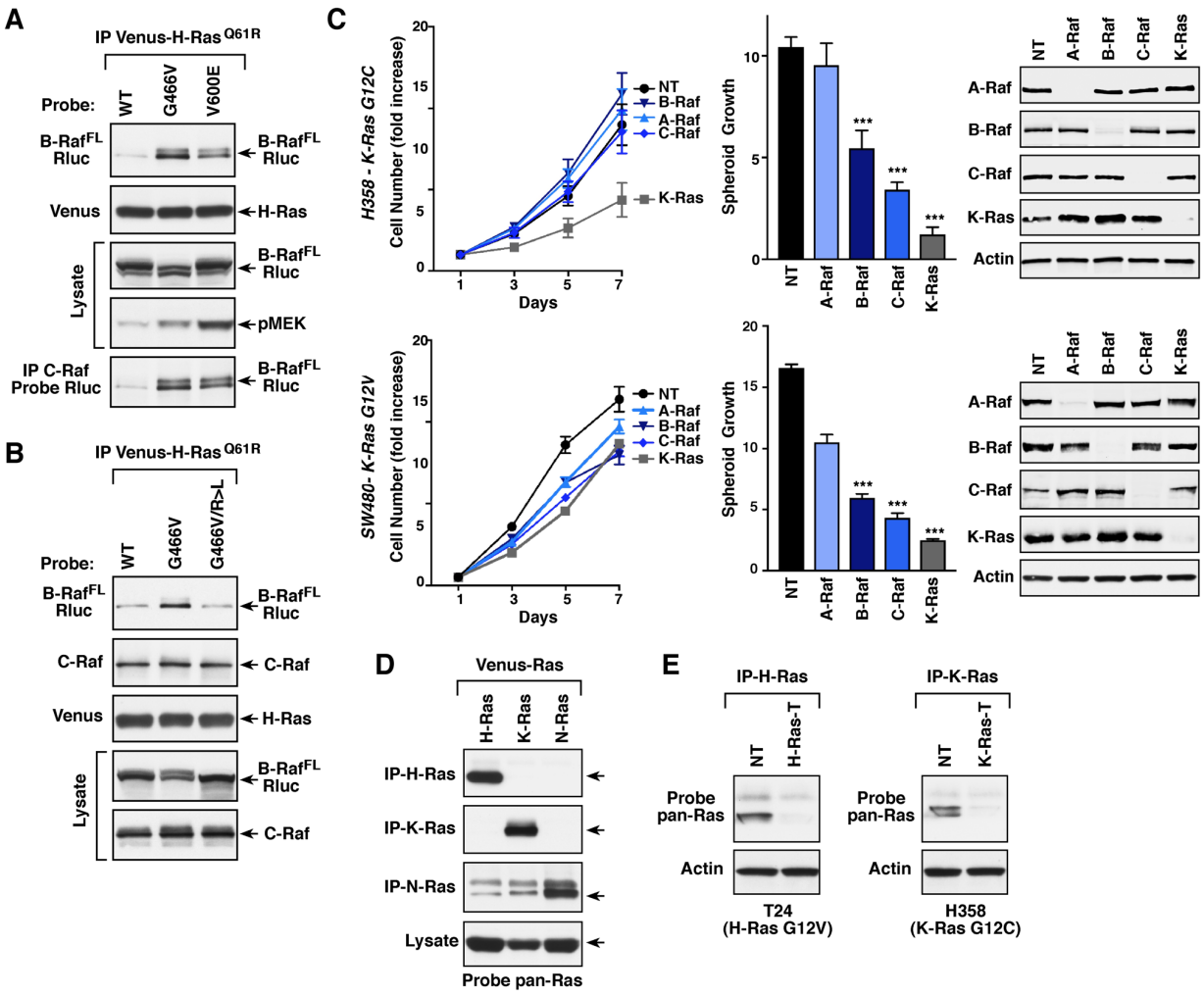

Figure S6, related to Figure 6. Elucidating C-Raf Functions in Ras-dependent Signaling

(A) Co-immunoprecipitation assays were performed comparing the ability of WT, G466V, or V600E-B-Raf^{FL}-Rluc proteins to interact with Venus-H-Ras^{Q61R}. Endogenous C-Raf was also immunoprecipitated and examined for dimerization with the B-Raf^{FL}-Rluc mutants. Lysates were monitored for pMEK and B-Raf-Rluc levels. (B) Co-immunoprecipitation assays were performed examining the interaction of WT, G466V or G466V/R>L B-Raf^{FL}-Rluc with Venus-H-Ras^{Q61R}. Co-immunoprecipitation of endogenous C-Raf with Venus-H-Ras^{Q61R} is also shown. (C) Human cancer cell lines harboring K-Ras mutations, H358 and SW480, were infected with lentiviruses expressing Cas9 and either a non-targeting sgRNA (NT) or sgRNAs targeting the *A-Raf*, *B-Raf*, *C-Raf*, or *K-Ras* gene. Cell lysates were examined for A-Raf, B-Raf, C-Raf, and K-Ras expression (right). Cells were assessed for 2D cell proliferation (left) and for 3D spheroid growth (middle). Data are

represented as mean +/-SD. ***$p < 0.001$. (D) Lysates of 293FT cells expressing Venus-tagged H-Ras^{G12V}, K-Ras^{G12V}, or N-Ras^{G12V} were each incubated with H-Ras, K-Ras, or N-Ras antibodies, following which the immune complexes were probed with pan-Ras antibodies in immunoblot analysis. These results indicate that the Ras antibodies are functional in immunoprecipitation assays and that the H-Ras and K-Ras antibodies specifically immunoprecipitate H-Ras and K-Ras respectively, whereas the N-Ras antibody exhibits some cross-reactivity with H-Ras and K-Ras. (E) Endogenous H-Ras was immunoprecipitated from lysates of control (NT) or H-Ras-depleted (H-Ras-T) T24 cells using the H-Ras antibody, and endogenous K-Ras was immunoprecipitated from lysates of control (NT) or K-Ras-depleted (K-Ras-T) H358 cells using the K-Ras antibody. The immune complexes were then probed with pan-Ras antibodies to further demonstrate the specificity of the H-Ras and K-Ras antibodies.