

1 **Intrinsic K-Ras dynamics:**

2 **A novel molecular dynamics data analysis method**

3 **shows causality between residue pairs**

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10

## 11 **Summary**

12 While mutant K-Ras is an important therapeutic target for human cancers, there are still no  
13 drugs that directly target it. Recent promising studies emphasize the significance of dynamics  
14 data to selectively target its active/inactive states. However, despite tremendous information  
15 on K-Ras, the direction of information flow in the allosteric regulation of its dynamics has not  
16 yet been elucidated. Here, we present a novel approach that identifies causality in correlated  
17 motions of proteins and apply it to K-Ras dynamics. Specifically, we analyze molecular  
18 dynamics simulations data and comprehensively investigate nucleotide-dependent intrinsic  
19 K-Ras activity. We show that GTP binding leads to characteristic residue correlations with  
20 relatively long decay times by stabilizing K-Ras motions. Furthermore, we identify for the first  
21 time driver-follower relationships of correlated motions in the regulation of K-Ras activity. Our  
22 results can be utilized for directly targeting mutant K-Ras in future studies.

## 23 **Introduction**

24 K-Ras is a small GTP-binding protein pivotal in cellular signaling. Somatic K-Ras mutations  
25 are among the most common activating cancer lesions, driving 71% of pancreas, 35% of  
26 colon and 17% of lung cancers (Slebos et al., 1990, Stephen et al., 2014, Forbes et al.,  
27 2015). Signaling through K-Ras is dependent on the bound nucleotide, where the GTP-  
28 bound state is active (on) while the GDP-bound state is inactive (off). In GTP-bound K-Ras,  
29 P-loop (residues 10-17), switch I (SI, residues 25-40) and switch II (SII, residues 60-74)  
30 regions make up the active site whose well-ordered conformations allow effector protein  
31 binding for protein signaling (Figure 1). However, oncogenic gain-of-function mutations  
32 impair GTP hydrolysis and freeze K-Ras in its active state (Vetter and Wittinghofer, 2001),  
33 causing uncontrollable cellular growth and evasion of apoptotic signals (Downward, 2003,  
34 Chen et al., 2013). Tumors driven by oncogenic K-Ras are often resistant to standard  
35 therapies and result in poor outcomes; they are also excluded from treatment with other  
36 targeted therapies, making mutant K-Ras an extremely high priority target in cancer  
37 treatment (Pao et al., 2005, Lievre et al., 2006). However, there are still no drugs in the clinic  
38 today that directly target mutant K-Ras.

39 Part of the challenge in oncogenic K-Ras inhibitor design has been due to structure analyses  
40 that suggest a lack of well-defined druggable sites on its surface (Zhang and Cheong, 2016).  
41 However, studies that have utilized protein dynamics data such as NMR and mass  
42 spectrometry have identified binding pockets on specific K-Ras oncogenic mutants and have  
43 attempted to stabilize their on or off conformational states (Taveras et al., 1997, Ostrem et  
44 al., 2013, Lim et al., 2014, Lito et al., 2016). Accumulating studies suggest that K-Ras  
45 proteins are in dynamic and flexible states and their distinct characteristics cannot be  
46 identified by structural studies alone (Patricelli et al., 2016, Ostrem et al., 2013, Lim et al.,  
47 2014, Singh et al., 2015, Grant et al., 2011b, Leshchiner et al., 2015, Lito et al., 2016). K-Ras  
48 dynamic activities in different conformational states, which can also change due to allosteric  
49 interactions between protein residues, also need to be quantified (Motlagh et al., 2014).

50 However, we still do not clearly understand the intra-molecular allosteric networks between  
51 distant sites on K-Ras (Marcus and Mattos, 2015). While such allosteric interaction sites  
52 have recently been discovered in its catalytic domain (Buhrman et al., 2010, Abankwa et al.,  
53 2010, Kearney et al., 2014), they remain largely understudied (Marcus and Mattos, 2015).  
54 Understanding allosteric interactions can present novel opportunities for small molecule  
55 targeting of mutant K-Ras conformations while sparing those of wild-type K-Ras, which first  
56 requires a deeper understanding of the intrinsic K-Ras dynamics.

57 In allosteric regulation of protein dynamics, correlated motions between protein residues are  
58 essential (Goodey and Benkovic, 2008, Kern and Zuiderweg, 2003, Wand, 2001). These  
59 motions enable the transfer of fluctuation information through the allosteric network  
60 (Kamberaj and van der Vaart, 2009), which inherently involves directionality, or “causality” of  
61 events (Guarnera and Berezovsky, 2016). If the motions of two residues are correlated, it  
62 would be extremely valuable to identify whether the motions of one residue drive the motions  
63 of the other. However, while correlation calculations indicate interaction (which is necessary  
64 for allosteric transitions), they are symmetric and do not reveal the direction of information  
65 flow. To understand the directionality (or causality) between the motions of two residues, we  
66 need to incorporate time-delayed correlation estimations in our analyses (Schreiber, 2000,  
67 Guarnera and Berezovsky, 2016).

68 Here, we introduce a novel method that enables identification of causality between all  
69 residue pairs of a protein using time-delayed correlation information. For this purpose, we  
70 first record residue fluctuations calculated at every time step of a molecular dynamics (MD)  
71 simulation as a time series, and then calculate the time-delayed correlation of a residue pair  
72 as the correlation between two time series where one is shifted in time relative to the other.  
73 We conclude that the fluctuations of a residue control and modify the fluctuations of the  
74 delayed one if we observe a significant correlation between the fluctuations of two residues  
75 with a positive time lag. This is a completely novel approach to the analysis of structure-  
76 function relations, as well as to understanding K-Ras dynamics. We demonstrate the

77 simplicity of computing time-correlation functions in studying protein dynamics by applying  
78 the method to study K-Ras. We specifically focus on K-Ras because of its clinical importance  
79 and the availability of a large body of data which enable the validation of our theoretical  
80 predictions. Note that while correlations between the fluctuations of residue pairs have  
81 already been shown in several Ras protein studies (Grant et al., 2009b, Lukman et al., 2010,  
82 Kapoor and Travesset, 2015), despite the significantly large body of literature on K-Ras,  
83 there has been surprisingly little attention on the role of causality (or directionality) in  
84 correlation dynamics of K-Ras conformations.

85 In summary, we present a comprehensive study of intrinsic K-Ras dynamics, including  
86 detailed analyses of causality between the motions of its residues. We first provide detailed,  
87 quantitative descriptions of both active and inactive K-Ras from extensive MD simulations.  
88 We use a statistical thermodynamics interpretation of fluctuation correlations to quantify K-  
89 Ras 'stiffening' upon activation. Using stiffness calculations jointly with measurements of  
90 reduced relative fluctuations, we define protein stability and show that K-Ras is more stable  
91 in active conformation. To investigate time-dependent characteristics of correlated motions,  
92 we map and compare correlated motion patterns of active and inactive K-Ras, then discuss  
93 the differences of decay times of correlations between the two K-Ras forms in detail. Our  
94 results show that inactive K-Ras is marked by a pronounced decrease in correlated motions  
95 of residues for shorter periods, while active K-Ras correlations have longer decay times. We  
96 analyze the ensuing events at the atomic scale. Finally, to enable a deeper understanding of  
97 K-Ras dynamics, we introduce the first causality calculations for K-Ras and identify specific  
98 driver and follower residues during protein simulations.

## 99 **Results and Discussion**

### 100 **Comparison of stiffness changes in active and inactive K-Ras**

101 **GTP binding increases K-Ras stiffness.** To understand how nucleotide binding effects K-  
102 Ras dynamics, we quantified changes in its 'stiffness' – a metric that inversely correlates with

103 residue pair fluctuations - upon GTP vs. GDP binding. For this purpose, we represented the  
104 interaction between two fluctuating residue pairs ( $i$  and  $j$ ) as a spring with a constant  $k_{ij}$ ,  
105 based on a statistical thermodynamics interpretation we previously developed (Erman,  
106 2015). Plotting this spring constant for every residue pair in both GTP- (Figure 2A) and GDP-  
107 bound (Figure 2B) K-Ras, we observe strong coordination in the fluctuations of GTP  
108 phosphate groups with those of K-Ras (Figure 2A).

109 To zoom in on and directly compare the effects of nucleotide binding on K-Ras stiffness, we  
110 simply calculated the differences in spring constant values between GTP- and GDP- bound  
111 K-Ras. In Figure 2C, red dots indicate that the differences are largely due to the stiffening  
112 effects of GTP-binding on residue pair fluctuations. Notice that Regions 1-3 in Figure 2C that  
113 correspond to secondary structures show significant increase in  $k_{ij}$  when GTP-bound.  
114 Furthermore, Region 1 corresponds to strong coordination of  $\beta 2$  and  $\beta 3$  motions, while  
115 Regions 2 and 3 correspond to increased stiffness of  $\beta 4$ - $\alpha 3$  and  $\alpha 4$ .

116 **Nucleotide binding affects spring constant of  $\alpha 2$  (SII).** We next investigated the effects of  
117 nucleotide binding on the spring constant of  $\alpha 2$  (SII), because previous studies have shown  
118 that stiffness increases when SII refolds into an  $\alpha$ -helical conformation through GTP binding  
119 (Noe et al., 2005). We calculated the spring constants of the two terminal residues of  $\alpha 2$  (A66  
120 and T74), which were  $0.10 \text{ kcal/mol}\cdot\text{A}^2$  (69.91 pN/nm) for active and  $0.04 \text{ kcal/mol}\cdot\text{A}^2$  (27.78  
121 pN/nm) for inactive K-Ras. Previous studies have utilized various experimental methods that  
122 have all led to spring constants within  $\sim 0.09$ - $1.15 \text{ kcal/mol}\cdot\text{A}^2$  (60–80 pN/nm) for helices  
123 (Adamovic et al., 2008, Howard, 2001). Our results for both forms of K-Ras are in the same  
124 order of magnitude. Note that for active K-Ras the  $\alpha 2$  spring constant is equalent to the  
125 characteristic spring constant of  $\alpha$ -helices, while it is lower in inactive form. Hence, our  
126 results validate and quantify earlier, qualitative observations of Noe *et. Al.* (Noe et al., 2005)  
127 that the  $\alpha 2$  spring constant reaches to the level of an  $\alpha$ -helix spring constant during GTP  
128 binding.

129 **Overall spring constant is higher in active complex.** To estimate global changes in  
130 stiffness in response to nucleotide binding, we calculated the overall spring constants  $k_{overall}$   
131 (*details in Methods*) of nucleotide-K-Ras complexes, which were 0.70 kcal/mol·Å<sup>2</sup> (481.75  
132 pN/nm) for GTP-bound, and 0.55 kcal/mol·Å<sup>2</sup> (385.12 pN/nm) for GDP-bound K-Ras. Both  
133 are of the same order of magnitude with an experimental study for another protein,  
134 myoglobin, which has an overall spring constant of ~300 pN/m (Rico et al., 2013, Zaccai,  
135 2000) pointing to an order of magnitude agreement of overall stiffnesses of proteins in  
136 general. In conclusion, GTP-binding makes K-Ras more rigid.

137 **Secondary structure motions show the strongest coordination with the rest of the**  
138 **protein.** Quantifying the spring constant based on fluctuations allows for analyzing how,  
139 analogous to a virtual spring, the fluctuations of a specific residue are coupled with  
140 fluctuations of rest of the protein. To discover residues whose fluctuations are in strong  
141 coordination with K-Ras fluctuations and how they change between the two states, we  
142 compared the mean spring constant  $\bar{k}_i$  of each residue  $i$ , for both active and inactive K-Ras  
143 (Figure 2D) as described in *Methods*. A large  $\bar{k}_i$  value indicates that the motions of residue  $i$   
144 are stiffly coupled with the motions of the protein; while a small  $\bar{k}_i$  value indicates that the  
145 motions of the  $i$ th residue and the protein are flexibly coupled. For simplicity, we categorized  
146 the significant mean spring constant  $\bar{k}_i$  values as highest, high and smallest (For details  
147 please see Table S1). In both states, the highest  $\bar{k}_i$  values are of  $\beta$ -strand residues  $\beta 4$ ,  $\beta 5$   
148 and  $\beta 6$ , showing the strongest coordination of their motions with K-Ras motions. Next, high  
149  $\bar{k}_i$  values of  $\beta 1$ , P-loop and  $\alpha 5$  residues indicate that their fluctuations are also strongly  
150 coupled with those of the protein. On the other hand, the smallest  $\bar{k}_i$  values belong to SII  
151 region in active and SI and SII regions in inactive K-Ras which show that their residue  
152 fluctuations are not correlated with the rest of the protein (Table S2). Since we have defined  
153 the stiffness metric as a signifier of a decrease in residue fluctuations, we provide a second  
154 line of proof that increased stiffness stabilizes dynamic fluctuations in both forms of K-Ras by  
155 using Root Mean Square Fluctuation (RMSF) graph (Figure S1). Clearly, the residues with

156 the smallest mean spring constant  $\bar{k}_i$  values from Figure 2D have the highest RMSF values  
157 in Figure S1 and vice versa.

158 As indicated in previous studies where NMR and Atomic Force Microscopy were used,  
159 protein stiffness depends on secondary structure (Zaccai, 2000, Rico et al., 2013), where  
160 loops contribute to structural flexibility and show large fluctuations, while  $\beta$ -strands and  $\alpha$ -  
161 helices provide mechanical stability and show small fluctuations (Rico et al., 2013). Our K-  
162 Ras results are consistent with these general observations. In addition, we observe stiff  
163 coupling of the fluctuations of the P-loop and the protein. This observation is important since  
164 P-loop is the phosphate binding site of K-Ras and connects  $\beta$ 1 and  $\alpha$ 1 (Figure 1). Although  
165 loops are often flexible regions of proteins and show higher fluctuations, in K-Ras, motions of  
166 P-loop residues are stiffly coupled to those of the protein, especially in active state ( $\bar{k}_i = 1.08$   
167 kcal/mol·A<sup>2</sup> for K-Ras-GTP,  $\bar{k}_i = 0.85$  kcal/mol·A<sup>2</sup> for K-Ras-GDP).

168 **The mean spring constant values of residues in  $\beta$ 2,  $\beta$ 3,  $\alpha$ 3 and switch regions –**  
169 **especially SI- are higher in active K-Ras than in inactive K-Ras.** Finally, we calculated  
170 mean spring constant differences between active and inactive K-Ras,  $\Delta \bar{k}_i$  ( $\bar{k}_i$  K-Ras-GTP -  $\bar{k}_i$  K-  
171 Ras-GDP). Figure 2E shows that the fluctuations of  $\beta$ 2 and  $\beta$ 3 terminal (D38 and D57) and  $\alpha$ 3  
172 center (D92-I93) residues are in stronger coordination with those of active K-Ras (vs. inactive  
173 K-Ras) ( $\Delta \bar{k}_i > 0.43$  kcal/mol·A<sup>2</sup>). Our results also indicate that although residues of switch  
174 regions have the smallest  $\bar{k}_i$  values in both forms, some of their  $\bar{k}_i$  values increase  
175 significantly in active form. In Figure 2E,  $\Delta \bar{k}_i$  ranges between 0.20-0.36 kcal/mol·A<sup>2</sup> for  
176 residues in SI (D30-R41) and 0.02-0.19 kcal/mol·A<sup>2</sup> for residues in SII (G60-T74). These  $\Delta \bar{k}_i$   
177 values show stiffer coupling of the motions of GTP-K-Ras with the motions of switch  
178 residues, especially SI (vs GDP-K-Ras). This result is important as SI includes the binding  
179 site to effector proteins which only bind to GTP-bound K-Ras when SI flexibility is reduced  
180 (Spoerner et al., 2010). Earlier studies that used NMR spectra and RMSF calculation also  
181 support our results that GTP binding reduces the flexibility of both SI and SII, especially SI



182 (Shima et al., 2010, Kapoor and Travasset, 2015). Our results improve on this information by  
183 showing that fluctuations of switch regions –notably SI- are more stiffly coupled with K-Ras-  
184 GTP fluctuations (Figure 2E).

### 185 **Comparison of residue pair correlations for active and inactive K-Ras**

186 To identify if the fluctuations of one residue are related to fluctuations of another residue, we  
187 calculated the correlations of all residue-residue pairs in both GTP- vs GDP bound K-Ras  
188 complexes. As expected, cross-correlation coefficient maps of K-Ras-GTP (Figure 3A) and  
189 K-Ras GDP (Figure 3B) exhibit different correlation characteristics. The most remarkable  
190 differences between Figure 3A and 3B belong to two parts: (i) the correlation of  $\alpha 1$ -S1 with  
191 L10- $\alpha 5$  and (ii) the correlations between  $\beta 2$  and  $\beta 3$ . Positive correlation patterns within these  
192 two parts are evident in K-Ras-GTP simulations, but absent in K-Ras GDP simulations. To  
193 provide comprehensive information on nucleotide-dependent K-Ras dynamics, we present  
194 these two remarkable results from correlation analyses (Figure 3) as well as sources of  
195 correlated motions (i.e. H-bonds) together in the following sections.

196 **The correlation of  $\alpha 1$ -S1 with L10- $\alpha 5$  in active K-Ras motions is due to three specific**  
197 **H-bonds.** MD simulations show that the correlation between  $\alpha 1$ -S1 and L10- $\alpha 5$  in the active  
198 form results from GTP binding to active site residues, which also form specific H bonds with  
199 other K-Ras residues and water. Based on the average number of H-bonds each residue  
200 forms throughout the simulation, we estimated that the nucleotides remain bound to active  
201 site residues S17, D30, D119 and K147, and that GTP-binding (vs. GDP) is more stable for  
202 S17 and D30 (Table S3). Furthermore, correlated motions of  $\alpha 1$ -S1 and L10- $\alpha 5$  in GTP-  
203 bound K-Ras originate specifically from three H-bonds: (i) A146-Q22, (ii) D30-GTP, (iii) D30-  
204 a water molecule. We observed a sustained H-bond between A146-Q22 during active but not  
205 in inactive complex simulation. This suggests that A146-Q22 interaction causes a strong  
206 relationship between L10 $\alpha 5$  (A146-D154) and  $\alpha 1$  (L19-I24) in active K-Ras (Figure 3A) with a  
207 correlation coefficient of 0.75, and a weak correlation coefficient of 0.28 for inactive K-Ras. At

208 the same time, the active site residue D30 forms an H-bond with the nucleotide in both active  
209 and inactive K-Ras, while it also binds to a water molecule only in the active form. However,  
210 the H-bond in the active form between D30(O)-GTP(O2A) is more permanent than the H-  
211 bond in the inactive form between D30(O)-GDP(O2').

212 **Since H-bond of D30-GTP is permanent, D30-GTP distance is invariant and their**  
213 **fluctuation correlations have longer decay times during K-Ras-GTP simulation.** We  
214 next combined cross-correlation results with the distance distribution of D30 and nucleotides  
215 and quantified the decay times of their correlations during MD simulations. In addition to  
216 more permanent binding of D30(O)-GTP(O2A), nucleotide-D30 distance distribution pattern  
217 is close to the normal distribution curve with a mean of a smaller value in active K-Ras  
218 (Figure 4A), with a correlation coefficient of 0.97. To quantify decay time of this correlation in  
219 both complexes, we first defined two “connectivity vectors”,  $\Delta R_{30-GTP}$  and  $\Delta R_{30-GDP}$ , between  
220 D30(O) and nucleotides. As illustrated in Figure 4B,  $\Delta R_{30-GTP}$  connects the starting point of  
221 fluctuation vector of  $\Delta R_{D30(O)}$  to end point of negative  $\Delta R_{GTP(O2A)}$ ;  $\Delta R_{30-GDP}$  starts from  $\Delta R_{D30(O)}$   
222 to negative  $\Delta R_{GDP(O2')}$ . We then calculated time-delayed autocorrelations of each connectivity  
223 vector throughout the MD simulations. The autocorrelation plot in Figure 4B summarizes the  
224 correlation of connectivity vectors at various time delays, where vector correlation  
225 coefficients are plotted with 1 ns delays at a time; slow decay of correlations in active K-Ras  
226 is clearly observed. Correlations decay to 1/e in about 3 ns for K-Ras-GDP (red line), vs. to  
227 ~10 ns for K-Ras-GTP (black line). One reason for this slow correlation decay is the H-bond,  
228 which binds D30 to a water molecule in active K-Ras. The O atom of D30 establishes an H-  
229 bond with the nearest water during 28% of the trajectory while it does not make any contact  
230 with waters when K-Ras is inactive.

231 **A continuous H-bond stabilizes  $\beta 2$ - $\beta 3$  distance and promotes longer decay times for**  
232  **$\beta 2$ - $\beta 3$  correlations during K-Ras-GTP simulation.**  $\beta 2$  and  $\beta 3$  are two parallel  $\beta$  strands  
233 located between SI and SII regions (Figure 5A). Due to the presence of a persistent H-bond  
234 between R41( $\beta 2$ )-D54( $\beta 3$ ) in K-Ras-GTP simulation, the peak value of  $R_{41-54}$  distribution

235 decreases (Figure 5B) and fluctuations of  $\beta 2$  and  $\beta 3$  become correlated (Figure 3A). The  
236 time-delayed autocorrelations of the vector  $\Delta R_{38-57}$  between their terminal residues D38 and  
237 D57 are presented in Figure 5C showing that  $\Delta R_{38-57}$  correlation decays much more slowly in  
238 active K-Ras.

### 239 **Causality of Correlated Motions**

240 Correlated motions of proteins often have a direction or causal relationship (Kamberaj and  
241 van der Vaart, 2009). Correlations in the fluctuations of two residues indicate interaction,  
242 which is necessary for allosteric transitions. However, this is not sufficient for understanding  
243 the dynamic phenomenon completely since these symmetric correlations do not contain  
244 information on driver and follower relationships. To deduce causality, time-delayed  
245 correlations need to be analyzed. Our trajectory analysis shows that the symmetry  
246 assumption on time-delayed correlations does not hold (Callen, 1985) for several residue  
247 pairs i.e., time delayed correlations of fluctuations of two atoms are not the same in forward  
248 or backwards in time. Our observation is supported by recent work (Kamberaj and van der  
249 Vaart, 2009) that identified causality in correlated motions from MD simulations using an  
250 information theory measure of transfer entropy. This work, in turn, was built on a study by  
251 Schreiber, who introduced the entropy transfer concept for fluctuating environments  
252 (Schreiber, 2000). We follow up on these ideas and introduce a new method to dissect time-  
253 dependent correlations of all residue pairs of a protein to identify driver and follower residues.  
254 For this purpose, we evaluate strong time-delayed ( $\tau=5\text{ns}$ ) correlations between residue  
255 pairs (defined as  $< -0.3$  or  $> 0.6$ ). The strongest causal relations are as follows (Figure 6):

256 **SII motions drive SI in active K-Ras.** SI-SII relationship is better understood by examining  
257 residues that drive their motions throughout the trajectory. Our causality calculations show  
258 that SI is driven by SII (Figure 6A and 7). We present time-delayed correlation plots of  
259 R68(SII) with V29(SI) (Figure 7A) and with P34(SI) (Figure 7B) for active K-Ras. Red curve  
260 shows that the fluctuations of R68 at time  $t$  affect the fluctuations of V29 at time  $t+\tau$ .

261 Fluctuation decay of K-Ras residues is in the order of 1ns. The red curve persists for time  
262 periods that are an order of magnitude longer. The reverse does not show a significant  
263 correlation: V29 does not correlate with later fluctuations of R68. Previously, a study reported  
264 that SI loop at residues 29–34 swings into the water using V29 and P34 as hinges during  
265 Ras inactivation (Noe et al., 2005). We improved on this information by calculating time-  
266 delayed correlations and identified that SII residues - especially R68 and D69- sustain active  
267 state conformation of SI by driving the motions of hinge residues V29 and P34. Another  
268 study also assessed the conformational transition of Ras from inactive to active state (Gorfe  
269 et al., 2008), where displacement of SII triggers the active state transition and SI follows SII  
270 after a lag time of multiple nanoseconds. Dominance of SII region motions was also  
271 observed in several studies (Clausen et al., 2015, Grant et al., 2009a). The nucleotide-bound  
272 form behavior is regulated by the relative arrangement of the two switches, rather than their  
273 individual conformations. We quantified this by verifying that SI fluctuations follow SII  
274 fluctuations in K-Ras-GTP. Since from an information theoretic point of view correlations are  
275 regarded as information sources, we conclude that information flows from SII to SI. The  
276 directionality originates from the differences in the characteristic decay times. The problem is  
277 therefore a problem of dynamics within few nanosecond time periods. Disruption of this flow  
278 is expected to interfere with the switch mechanism function, which is the basis of K-Ras  
279 activity.

280  **$\alpha$ 3 and Loop 7 (L7) motions drive switch region (SI & SII) motions in active K-Ras**  
281 **(Figure 6B).** Fluctuations of the helical dimer interface residues of  $\alpha$ 3, E98 and R102  
282 (Muratcioglu et al., 2015) drive fluctuations of A66 ( $\alpha$ 2; SII), as shown in Figures 8C and 8D.  
283 Additionally, helical dimer interface residue S106 (L7) drives the motion of Y71 ( $\alpha$ 2; SII)  
284 (Figure 8E). On the other hand, fluctuations of R102 ( $\alpha$ 3) and S106 (L7) drive SI residues  
285 N26, D30, Y32 (Figures 8F to 8H).

286 Correlated motions of  $\alpha$ 2 and  $\alpha$ 3-L7 have been described in other studies, which also  
287 emphasized the necessity of understanding their effect on protein function (Grant et al.,

288 2009b, Clausen et al., 2015, Grant et al., 2011b). We contribute to this knowledge by  
289 identifying their cause and effect relations. Furthermore, in previous studies, starting from the  
290 allosteric interaction between  $\alpha 2$  and  $\alpha 3$ -L7, a novel ligand binding pocket, termed p3, which  
291 includes residues of L7 was defined and targeted for lead generation (Grant et al., 2011b,  
292 Spoerner et al., 2005, Rosnizeck et al., 2010). It was reported that ligand binding to p3  
293 pocket weakens effector protein binding by allosterically stabilizing Ras effector binding site  
294 (SI). Another proposed allosteric mechanism is that ligand binding to p3 pocket changes the  
295 switch region conformation. Our results implicate the role of allosteric modulation of ligand  
296 binding, which may freeze the fluctuations of L7 and stabilize SI motions. This is based on  
297 our finding that motions of effector binding site (D30-Y32) are driven by S106 (L7).

298  **$\beta 2$ - $\beta 3$  are both drivers and followers in active K-Ras.** Causality calculations suggest the  
299 following information flow in fluctuations: ILE21-GLN22 ( $\alpha 1$ ) drives  $\beta 2$ - $\beta 3$  (Figure 6 and S2);  
300 which drives Y157 ( $\alpha 5$ ), Q61 (SII) and T74 (SII) (Figure 6 and S3, with details in Table S4).  
301 Specifically, the differences in the characteristic decay times in Figures S3A-B demonstrate  
302 that information flows from  $\beta 2$ - $\beta 3$  to Y157 ( $\alpha 5$ ). These findings improve on the previous  
303 observations of Abankwa et al. where they defined  $\beta 2$ - $\beta 3$  and  $\alpha 5$  as a novel conformational  
304 switch (Abankwa et al., 2008). Most importantly, we showed that Q61 (SII) motions follow  
305 E49 motions ( $\beta 2$ - $\beta 3$ ) (Figure S3D). Abankwa et al. also observed that mutations in D47-E49  
306 cause hyperactive Ras. Our findings support this too by showing that fluctuations of E49 of  
307 the wild type cause fluctuations of the catalytic residue Q61 within SII, whose proper  
308 positioning is essential for effective catalysis (Ito et al., 1997). Based on these results, we  
309 suggest that mutations in D47-E49 region may alter E49 fluctuations that cause improper  
310 Q61 fluctuations. Therefore, GTP catalysis is disrupted which results in constitutively active  
311 K-Ras.

312 **Conclusions**

313 We present a novel approach that combines several distinct analysis methods to quantify in  
314 detail dynamics of GTP and GDP bound K-Ras, for which a significant amount of  
315 experimental and theoretical data already exists in the literature to test our predictions.  
316 Oncogenic K-Ras is an extremely high priority drug target in cancer treatment. In order to  
317 develop new direct inhibitors that selectively bind to mutant K-Ras conformations while  
318 sparing those of WT K-Ras, it is necessary to first understand the dynamic activity of the WT  
319 protein in detail. To evaluate the nucleotide binding dependent changes in K-Ras stability, we  
320 used stiffness and RMSF calculations and proved that GTP binding rigidifies and hence  
321 stabilizes K-Ras motions. These results are in agreement with previous experimental and  
322 computational K-Ras studies (Kapoor and Travasset, 2015, Raimondi et al., 2011, Diaz et  
323 al., 1995).

324 Our calculations that use stiffness, RMSF and correlation graphs (Figure 1-2, S1) confirm  
325 that GTP binding increases K-Ras stiffness and thereby decreases fluctuation amplitudes,  
326 leading to distinct correlation patterns. These striking changes in GTP bound-K-Ras  
327 dynamics enable its GTPase activity. Note that this nucleotide exchange is the first step in  
328 active to inactive transition (Prakash et al., 2012, Prakash et al., 2015, Zhang et al., 2012,  
329 Buhrman et al., 2011, Prakash and Gorfe, 2013, Grant et al., 2011a, Edreira et al., 2009).

330 Overall, our results support the well-established allosteric nature of K-Ras activation  
331 (Prakash and Gorfe, 2013), which has been suggested to play an important role in GTPase  
332 activity (Grant et al., 2010). Although correlated fluctuations are necessary for allosteric  
333 information flow, their longer correlation decay times are also of crucial importance for  
334 complete allosteric transition. We calculated time-dependent autocorrelations of fluctuation  
335 vectors between residue pairs and discovered that correlations of K-Ras-GTP are stronger  
336 and persist for longer correlation times during simulations. Their persistency may allow  
337 complete allosteric information flow in K-Ras-GTP.

338 We broadened our analysis to quantify causality in allosteric regulation of K-Ras function.  
339 The most important results from our study are on causality. We applied a simple but powerful

340 method that we defined as *time-delayed correlation* into protein dynamics. To understand K-  
341 Ras dynamics, we investigated whether fluctuations of any residue caused fluctuations of  
342 another. Our results revealed the information flow in K-Ras switch mechanism and that SII  
343 fluctuations drive SI fluctuations. This prediction is an essential validation of our approach,  
344 since the dominance of SII motions over SI motions was observed in previous experimental  
345 and computational studies (Clausen et al., 2015, Grant et al., 2009a). Surprisingly, in addition  
346 to the canonical switch mechanism, our algorithm also revealed causality relations in the  
347 novel switch mechanism that includes  $\beta 2$ – $\beta 3$  and  $\alpha 5$ , where  $\beta 2$ – $\beta 3$  motions drive  $\alpha 5$ .  
348 Moreover, fluctuations of  $\alpha 3$ -L7 drive fluctuations of SI and SII. Interestingly, previous studies  
349 reported that Ras effector binding site (SI) is allosterically stabilized by ligand binding into a  
350 novel pocket that includes L7 (Grant et al., 2011b, Spoerner et al., 2005, Rosnizeck et al.,  
351 2010). Our results explain the allosteric effect of ligand binding on SI motions by showing the  
352 information flow from L7 to SI.

353 Note that functionally, the identified driver and follower sites do not show an enrichment trend  
354 in oncogenic mutations observed in human cancers (from 2266 missense K-Ras mutations  
355 observed in all cancers within cBioPortal [www.cbioportal.org](http://www.cbioportal.org) on July 28, 2016). However, the  
356 motions of residue Q61 (SII region), which is the second most frequently mutated residue in  
357 cancer patients (113/2266 missense mutations) are driven by those of E49 ( $\beta 3$ ). While there  
358 are no known oncogenic mutations of residue E49, experiments have shown that mutating  
359 E49 leads to hyperactive Ras (Abankwa et al., 2008), consistent with what we would expect  
360 from a driver of Q61 motions, which also causes the same effect.

361 Our ongoing research on mutated K-Ras suggests deviations in dynamics from that of WT K-  
362 Ras. Any such investigation would first necessitate a deep understanding of the causal  
363 relationships in intrinsic K-Ras motions. Understanding intrinsic WT dynamics is imperative  
364 and serves as a necessary reference. Our objective behind this detailed analysis is to  
365 provide such a reference for any future mutant K-Ras studies. It would be of interest to  
366 identify how these findings change when there is a mutation on the protein. Only after

367 identifying these differences can we discover molecules that can eliminate the unfavorable  
368 changes caused by the mutations.

369 The computational tools we introduce in the present work are easily applicable to the  
370 analysis of simulation data from different proteins to understand causality in their allosteric  
371 regulations which can then be utilized in drug discovery. From this perspective, our approach  
372 sets a novel paradigm for drug design that directs attention to changes in protein dynamics.  
373 The latter is in close relation to changes in protein function whose restoration to normal is the  
374 target of all drug design activities.

## 375 **Experimental Procedures**

### 376 **MD Simulations**

377 We performed all-atom MD simulations for both Mg<sup>+2</sup>GDP- and Mg<sup>+2</sup>GTP-bound K-Ras. As  
378 the initial point of all simulations, we used the crystal structure corresponding to PDB ID  
379 4OBE (K-Ras-GDP). For K-Ras-GTP structure, we simply substituted the GDP of K-Ras-  
380 GDP structure with GTP using Discovery Studio 4.5 software (BIOVIA, 2015). We solvated  
381 each protein in a water box (TIP3 water) where buffering distance between box edges and  
382 protein was 12 Å. We applied periodic boundary conditions and added counter-ions to  
383 neutralize the system. We used a 2 fs time-step with a 12Å cutoff for Van der Waals  
384 interactions and full particle-mesh Ewald electrostatics. We carried out all computations in  
385 dynamics procedure for an N, P (1 atm), T (310K) ensemble. We used NAMD 2.10 with  
386 AMBER ff99SB and general amber force fields (GAFF). We obtained parameters of GTP and  
387 GDP (see Supplemental Experimental Procedures). The initial system energy was first  
388 minimized for 10,000 steps, followed by 10,000 steps for equilibration. After equilibration, we  
389 performed 300 ns simulations. Atomic coordinates  $\hat{R}$  of all atoms were saved every 1 ps. To  
390 eliminate all rotational and translational motions, we aligned the trajectories to the initial  
391 structure by using VMD software 1.9.2 (Humphrey et al., 1996). We visualized trajectories  
392 using VMD.



### 393 **Stiffness**

394 We quantified nucleotide-bound K-Ras stiffness using a statistical thermodynamics  
395 interpretation of fluctuation correlations (Erman, 2015). We assumed that the interaction  
396 between two fluctuating residues  $i$  and  $j$  can be represented by a spring, where the spring  
397 constant follows from the Gaussian Network Model (GNM) (Haliloglu et al., 1997):  $k_{ij} =$   
398  $\frac{k_B T}{\langle(\Delta R_i)^2\rangle - 2\langle\Delta R_i \Delta R_j\rangle + \langle(\Delta R_j)^2\rangle}$  where  $\Delta R_i$  is the instantaneous fluctuation of one end of the rod,  $\Delta R_j$   
399 is the fluctuation of the other end (Details in Supplementary),  $k_B$  is the Boltzmann constant  
400 and  $T$  is the absolute temperature. The spring constant has dimensions of force/length. In  
401 GNM spring definition, each residue  $i$  is attached to  $N-1$  other residues via  $N-1$  springs  
402 (Haliloglu et al., 1997). Thus, how stiffly a residue  $i$  is attached to a protein can be quantified  
403 by  $\bar{k}_i = \sum_j k_{ij} / N - 1$  where  $\bar{k}_i$  is the mean spring constant for a residue  $i$ . For stiffness  
404 estimates of the complete complexes, we define an overall stiffness parameter  $k_{overall}$  by the  
405 expression  $k_{overall} = \sum_{j>i} k_{ij}$ . To estimate the stiffness differences in active versus inactive K-  
406 Ras, we calculated  $\bar{k}_i$  for each residue and  $k_{overall}$  for the protein for both states.

### 407 **Stability**

408 We defined the stability of an interacting system of residues as the joint state of reduced  
409 RMSF and increased interaction stiffness. RMSF relates to the magnitude of fluctuations of  
410 individual residues and stiffness relates to the distance between two residues and therefore  
411 they are two independent quantities. (For details see Supplemental Experimental  
412 Procedures). A small RMSF and a high stiffness denote increased stability.

### 413 **Distance distributions between residue pairs**

414 We calculated the distance between two residues ( $i,j$ ) as  $R_{ij} = \sqrt{(R_i(t) - R_j(t))^2}$ . Residue  
415 pair distance distributions  $W(R_{ij})$  were calculated by dividing the maximum distance between

416 the pair into small bins and counting the number of observed distances in each bin. All  
 417 distributions were normalized.

418 **Time independent correlations (cross-correlation coefficient map):** Correlations intrinsic  
 419 to K-Ras structure are defined by the cross-correlation coefficient map,  $C(\Delta R_i, \Delta R_j)$ :

$$420 \quad C_{ij} = C(\Delta R_i, \Delta R_j) = \frac{\langle \Delta R_i(t) \cdot \Delta R_j(t) \rangle}{\langle (\Delta R_i(t))^2 \rangle^{1/2} \langle (\Delta R_j(t))^2 \rangle^{1/2}} = \frac{\sum_{t=1}^{N_t-\tau} \Delta R_i(t) \cdot \Delta R_j(t)}{\left[ \sum_{t=1}^{N_t-\tau} (\Delta R_i^2(t)) \right]^{1/2} \left[ \sum_{t=1}^{N_t-\tau} (\Delta R_j^2(t)) \right]^{1/2}}$$

421 where  $\cdot$  denotes the dot product. Correlation varies between -1 and 1. If motions of two  
 422 atoms are independent, then  $\langle \Delta R_i(t) \cdot \Delta R_j(t) \rangle = 0$  and  $C_{ij} = 0$ . If the atoms always move in  
 423 parallel in the same direction, then they are perfectly positively correlated, and  $C_{ij} = 1$ . If they  
 424 always move in parallel in opposite directions, they are perfectly negatively correlated, and  
 425  $C_{ij} = -1$ . Cross-correlation coefficients lie in the range of  $-1 \leq C_{ij} \leq 1$ .

426 **Time delayed correlations, mobility and causality**

427 Time-delayed correlation of two fluctuations is defined by:

$$C_{ij}(\tau) = \frac{\langle \Delta R_i(t) \cdot \Delta R_j(t + \tau) \rangle}{C_{ij}} = \frac{\sum_{t=1}^{N_t-\tau} \Delta R_i(t) \cdot \Delta R_j(t + \tau)}{\left[ \sum_{t=1}^{N_t-\tau} (\Delta R_i^2(t)) \right]^{1/2} \left[ \sum_{t=1}^{N_t-\tau} (\Delta R_j^2(t + \tau)) \right]^{1/2}}$$

428 where  $C_{ij}(\tau)$  represents the correlation of  $\Delta R_j$  at time  $t + \tau$  and earlier values of  $\Delta R_i$  at time  $t$ .  
 429 Similarly, if indices are exchanged, then  $C_{ji}(\tau)$  represents the correlations of  $\Delta R_i$  at time  $t + \tau$   
 430 with earlier values of  $\Delta R_j$  at time  $t$ . If the fluctuations of residue  $i$  drive the fluctuations of  
 431 residue  $j$ , then  $C_{ij}(\tau) > C_{ji}(\tau)$ . If  $C_{ji}(\tau) > C_{ij}(\tau)$ , residue  $j$  drives residue  $i$  because the  
 432 fluctuation  $\Delta R_j$  at time  $t$  is correlated with future fluctuations of  $\Delta R_i$ . However, at  $\tau=0$ , the  
 433 equality  $C_{ij}(0)=C_{ji}(0)$  holds.

434 Note that time-delayed autocorrelation  $C_{ii}(\tau)$  is the correlation of the trajectory with its own  
 435 past and future coordinates. If autocorrelation is large, this could correspond to a specific

436 form of “persistence”, a tendency for a system to remain in the same state from one  
437 observation to the next.

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442 Medicine at Mount Sinai.

### 443 **Figure Legends**

444 **Figure 1. Crystal structure of wild-type K-Ras protein in GTP-bound state (PDB:**  
445 **4OBE).** A) K-Ras structure ribbon representation with secondary structures in blue for  $\alpha$ -  
446 helices and green for  $\beta$ -sheets. B) Schematic of K-Ras sequences (residues 1-169).  
447 Functional regions are in same color used in K-Ras structure in A.

448 **Figure 2. Stiffness results for GTP- and GDP-bound K-Ras and their difference.** In  
449 panels A and B, both axes marks 1-169 represent the residue C $\alpha$  atoms of K-Ras and marks  
450 170-on represent GDP and GTP nucleotide heavy atoms, respectively with  $k_{ij} > 1.5$   
451 kcal/mol·A<sup>2</sup>. (A)  $k_{ij}$  for K-Ras-GTP. Atoms 170-181 are the  $\gamma$ ,  $\beta$ ,  $\alpha$ -phosphate groups and 182-  
452 201 are the guanine atoms of GTP. (B)  $k_{ij}$  for K-Ras-GDP. Atoms 170-178 are the  $\beta$  and  $\alpha$   
453 phosphate groups and 178-197 are the guanine atoms of GDP. (C) Difference between  
454 active and inactive K-Ras  $k_{ij}$  values. Red regions are stiffer in K-Ras-GTP ( $k_{ij}$  values of K-  
455 Ras-GTP are greater than K-Ras-GDP at least 0.75 kcal/mol·A<sup>2</sup>) and blue regions are stiffer  
456 in K-Ras-GDP ( $k_{ij}$  values of K-Ras-GDP are greater than K-Ras-GTP at least 0.75  
457 kcal/mol·A<sup>2</sup>). (D) Mean spring constants  $\bar{k}_i$  for the GTP and GDP bound states. (E) Mean  
458 spring constant differences  $\Delta\bar{k}_i$  for the GTP and GDP bound states. Positive values  
459 correspond to larger mean stiffness in K-Ras-GTP.

460 **Figure 3. Cross-correlation coefficient maps for GTP and GDP bound states.** Red dots  
461 show positive correlations ( $1 \geq C(\Delta R_i, \Delta R_j) \geq 0.6$ ) and blue dots show negative correlations ( $-$   
462  $0.45 \geq C(\Delta R_i, \Delta R_j) \geq -1$ ). Residues indexed 1-169 belong to K-Ras. (A) Correlated  
463 fluctuations of K-Ras-GTP. Indices between 170-201 refer to GTP heavy atoms (182-201 are  
464 guanine atoms). (B) Correlated fluctuations of K-Ras-GDP. Indices between 170-197 refer to  
465 GDP heavy atoms (178-197 are guanine atoms).

466 **Figure 4. D30-GTP distance is more stable than that of D30-GDP. Fluctuations of**  
467 **D30(O) to GTP(O2A) “connecting vector” are persistently correlated.** (A) Distance  
468 distribution between D30 and connecting O atoms of GTP (black) and GDP (red) (B) Time  
469 delayed autocorrelations for the vector connecting Oxygen atom of D30 to O2A of GTP  
470 (black curve) and O2' of GDP (red curve). X-axis is the time delay ( $\tau$ ) and Y axis is the time  
471 delayed autocorrelation of the vector for  $\tau$ .

472 **Figure 5. Correlation of  $\beta 2$  and  $\beta 3$  fluctuations is persistent in active K-Ras.** (A)  
473 Locations of R41 ( $\beta 2$ ) and D54 ( $\beta 3$ ) relative to SI & SII. (B) Distance distribution between Ca  
474 atoms of R41 and D54 in K-Ras-GTP (black) and K-Ras-GDP (red). Distance values  
475 between R41 ( $\beta 2$ ) and D54 ( $\beta 3$ ) populate at 3.90 Å during GTP binding whereas these  
476 distance values populate at 5.46 Å for GDP-bound K-Ras. (C) Time delayed autocorrelations  
477 for the fluctuations of the vector from D38 (Ca) to D57 (Ca).

478 **Figure 6. Causality relations in active K-Ras motions.** Directionality in causal  
479 relationships is illustrated with arrows. Arrows start from driver residues and end at follower  
480 residues. Both residue types are represented with yellow spheres and marked with their  
481 residue numbers. The secondary structures they belong to are in turquoise. A) R68 (SII)  
482 drives V29 and P34(SI). B) E98 and R102 ( $\alpha 3$ ) drive A66 ( $\alpha 2$ ; SII). S106 (L7) drives Y71 ( $\alpha 2$ ;  
483 SII). R102 ( $\alpha 3$ ) drives N26 and Y32(SI). S106 (L7) drives D30. C) ILE21-GLN22 ( $\alpha 1$ ) drives  
484  $\beta 2$ - $\beta 3$ . D) I46 and D47 ( **$\beta 2$ - $\beta 3$** ) drive Y157 ( $\alpha 5$ ).

485 **Figure 7. SII fluctuations drive SI fluctuations;  $\alpha$ 3-L7 motions drive switch region (SI &**  
486 **SII) motions in K-Ras-GTP.** Red curves for  $\langle \Delta R_i(t) \Delta R_j(t + \tau) \rangle$  show that the fluctuations of  
487 residue  $i$  at time  $t$  affect the fluctuations of residue  $j$  at a later time  $t + \tau$ . (A) R68 (SII) drives  
488 V29(SI). (B) R68 drives P34(SI). (C) E98( $\alpha$ 3) drives A66 ( $\alpha$ 2; SII). (D) R102 ( $\alpha$ 3) drives A66.  
489 (E) S106 (L7) drives Y71 ( $\alpha$ 2; SII). (F) R102 ( $\alpha$ 3) drives N26(SI). (G) R102 drives Y32(SI).  
490 (H) S106 (L7) drives D30(SI).

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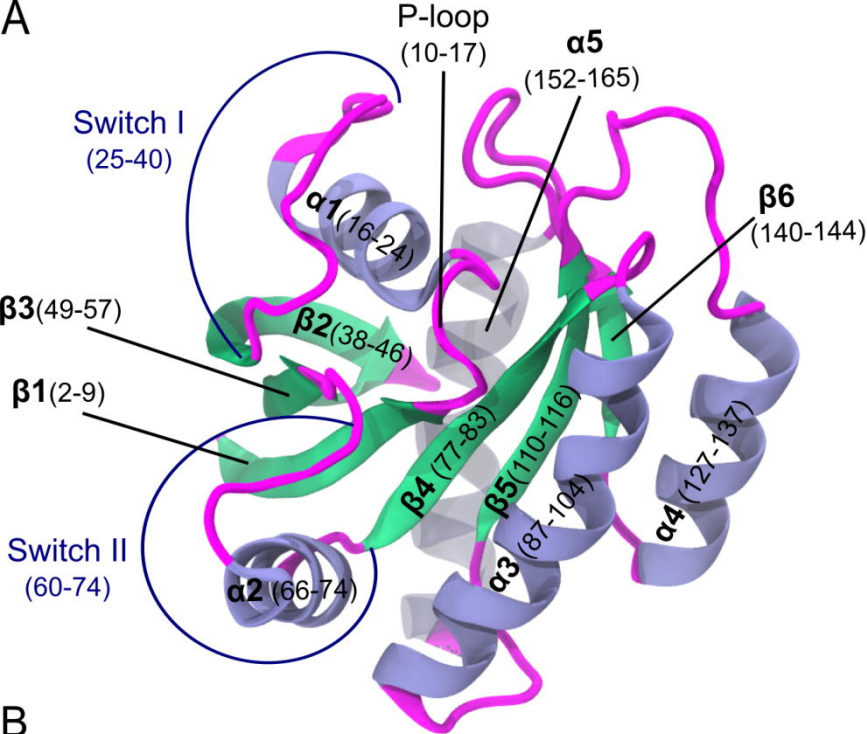
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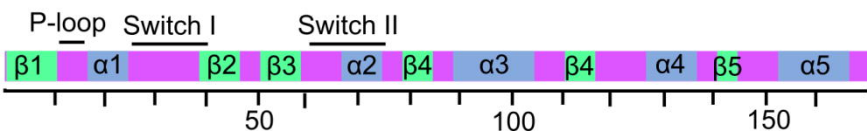
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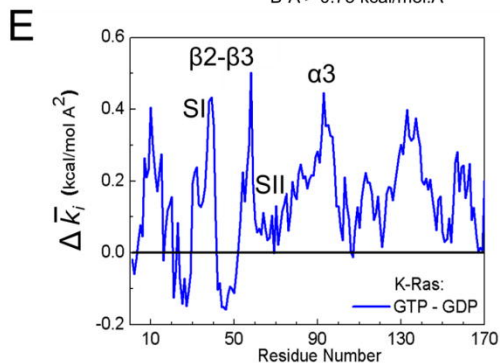
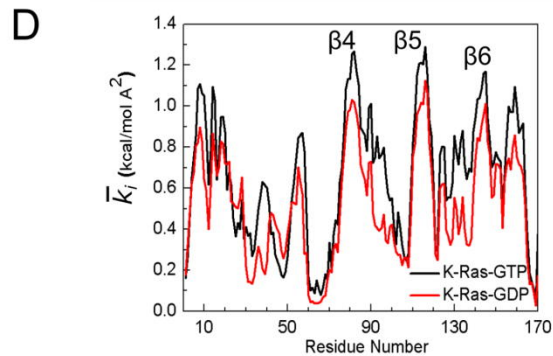
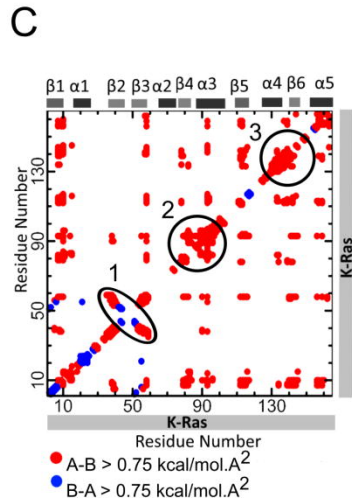
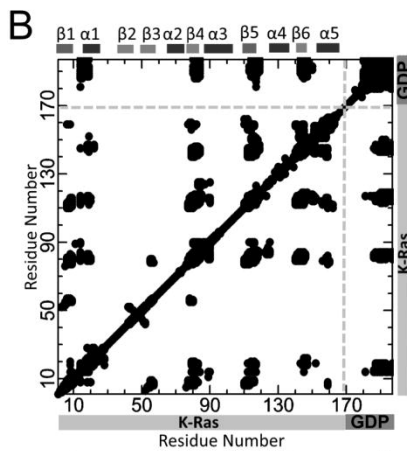
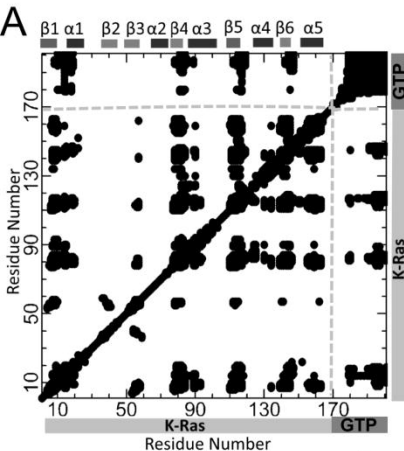


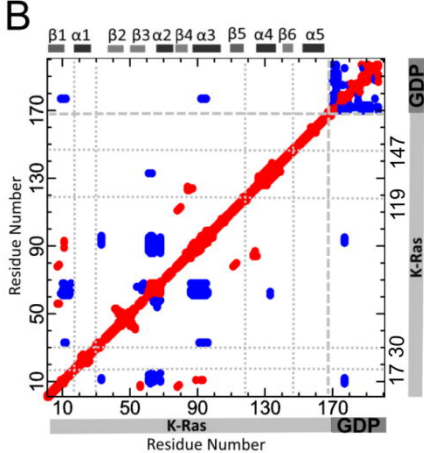
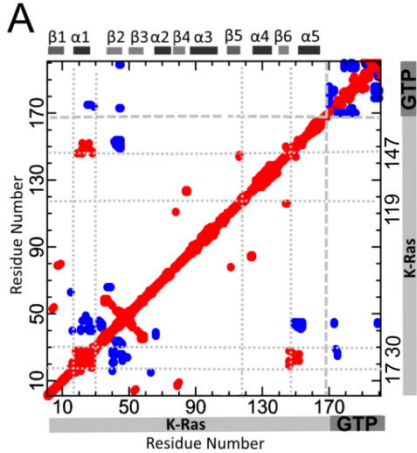
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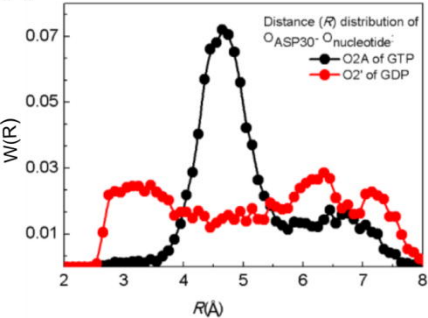






●  $1 \geq C(\Delta R_i, \Delta R_j) \geq 0.6$

●  $-0.45 \geq C(\Delta R_i, \Delta R_j) \geq -1$

**A****B**